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HYPOXIA-INDUCIBLE FACTOR 2*α* AND RELATED MEDIATORS IN THE PROGRESSION OF CHRONIC LIVER DISEASES

STUDENT: ELISABETTA MORELLO

TUTOR: MAURIZIO PAROLA

PhD COORDINATOR: GIUSEPPE SAGLIO

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INTRODUCTION

1. HYPOXIA

Oxygen (O_2) is a key molecular ingredient of life because of its utilization in the process of respiration. Tissue oxygenation represents a steady state based on O_2 consumption, primarily by mitochondrial oxidative phosphorylation where O_2 serves as the final electron acceptor for mitochondrial cytochrome c oxidase, which, in turn, represents the terminal functional element of the mitochondrial multicomponent NADH dehydrogenase enzymatic complex, which is able to catalyze the four electron reduction of O_2. Oxidoreduction energy of mitochondrial electron transport is then eventually converted to the high-energy phosphate bond of adenosine triphosphate (ATP), which is a source of energy necessary to maintain cellular viability. Under normoxic conditions then any cell continuously maintain a high and constant ratio of cellular ATP/ADP ratio in order to survive and operate the own specific functions. Low levels of oxygen in tissues (i.e., hypoxia), can be the consequence of a number of pathophysiological conditions, such as atherosclerosis, obstructive sleep apnea, mountain sickness, ischemic diseases and cancer, but can also occur in physiological processes, such as embryonic development (Gilany & Vafakhah, 2010). Hypoxia by itself can have several consequences on cellular functions, including either the chance to mount adaptative responses leading to cell survival or the risk of generating reactive oxygen species (ROS) able to react with cellular macromolecules and alter their biochemical or physical properties, possibly resulting in cell dysfunction or death (Semenza, 2012).

The normal O_2 concentration to which cells in the human body are exposed varies from ~21% [corresponding to a partial pressure (PO_2) of~150 mm Hg at sea level] in the upper airway to ~1% at the cortico-medullary junction of the kidney. The physiological tissue distribution of oxygen occurs as a result of progressive consumption of the oxygen in the blood circulation as it passes through different organs. The oxygen partial pressure of inspired air drops progressively first in the lungs; in part due to water vapor and diffusion, then in the blood flowing from the alveolar capillaries that carry the oxygen, at a pO_2 of around 104 mm Hg, towards organs and tissues for their oxygenation. A further drop in the pO_2 is observed in the venous system. The diffusion distance of oxygen in a tissue is

approximately 100–200 μm and an oxygen partial pressure of almost zero has been reported at only 100 μm from blood vessel (Brahimi-Horn & Pouysségur, 2007).

Complex homeostatic mechanisms serve to maintain the cellular O_2 concentration within a narrow range in vivo. Hypoxia response can be divided in different time scales, including an acute, in which rapid but transient responses are mediated through post translational modification of existing proteins, and a chronic phase, in which delayed but durable changes are mediated through altered gene transcription and protein synthesis. Finally, hypoxia can be systemic, as in the case of ascent to high altitude, or local, as in the case of myocardial ischemia associated with coronary artery disease (CAD) (Semenza, 2007).

2. OXYGEN SENSING AND HOMEOSTASIS

Organisms have evolved a complex array of mechanisms to sustain the supply of oxygen to the cells of the body and to protect themselves in the event that oxygen availability becomes limiting. The ability to sense the level of O_2 is therefore a fundamental requirement for survival of multicellular organisms. Well-known examples of specialized oxygen sensing systems include the carotid bodies (CB), the "sensory organs" for monitoring arterial blood O_2 levels. This arterial chemoreceptors signal the respiratory system to increase the level of alveolar ventilation when arterial partial pressure of O_2 decreases (Guzy & Schumacker, 2006). The response of the CB to hypoxia is notable for its speed and sensitivity: a 20% reduction in arterial PO_2 is sufficient to induce a response within seconds. Glomus (type I) cells are excitable cells in the CB that depolarize in response to hypoxemia. Axons from glomus cells course through the carotid sinus nerve (CSN) to the nucleus tractus solitarius, from which reflex arcs project to: the diaphragm, to increase respiratory rate; the heart, to increase heart rate; and via the rostral ventro-lateral medulla and sympathetic nervous system, to the adrenal medulla (AM), to stimulate the secretion of catecholamines (epinephrine and norepinephrine) that increase blood pressure. These cardiovascular and respiratory adaptations increase cardiac output and ventilation, respectively, thereby increasing the capture of O_2 by red blood cells for delivery throughout the body. O_2 sensing/signal transduction in the CB involves two gas messengers, CO and H_2S: under normoxic conditions, CO levels are high, H_2S levels are low, and carotid sinus nerve activity is low; in contrast, hypoxia leads to decreased CO

production and increased H_2S production, which induces CB cell depolarization and increased carotid sinus nerve activity (Samanta et al., 2017).

Given that the carotid body responds to hypoxemia within a few seconds, it is likely that the transduction process involves changes in existing proteins rather than de novo protein synthesis. Prolonged hypoxia initiates a series of physiological responses over a time scale of hours to days to maintain O_2 homeostasis. Examples include increased red blood cell production, formation of new blood vessels, and metabolic re-programming of cells (Prabhakar & Semenza, 2015).

3. HYPOXIA-INDUCIBLE FACTORS (HIFs)

Under chronic hypoxia, changes in the gene expression are regulated by the hypoxia-inducible factor (HIF) family of transcription factors. HIFs are basic helix-loop-helix DNA binding proteins of the ^{P}ER-^{A}RNT-^{S}IM family (bHLH-PAS). These transcription factors function as heterodimers composed of an oxygen-regulated α subunit (HIF-α) and a constitutively expressed β subunit. In mammals, the alpha subunits are encoded by three genes: HIF-1α, EPAS1 (also known as HIF-2α), and HIF-3α. Under conditions of normal oxygen tension, the α-subunits are hydroxylated at key proline and asparagine residues, which inhibits their transactivation function and targets them for proteasomal degradation. The HIF-1β subunits, also known as aryl hydrocarbon receptor nuclear translocators (ARNT), are encoded by two genes ARNT1 and ARNT2. HIF-1β is stably expressed and is also an obligate partner for the aryl hydrocarbon receptor (AHR) (Dengler et al., 2014).

Upon hypoxia, the HIF-α -subunits are stabilized and accumulate in the nucleus, where they dimerize with HIF-1β, allowing them to bind to DNA and stimulate the transcription of their target genes. This allows the activation of genes essential in the adaptive response to hypoxia, including pathways that decrease the cellular demand for O_2 and increase O_2 delivery. For example, the first HIF target gene identified was erythropoietin (EPO), a glycoprotein hormone that stimulates the generation of new red blood cells, thereby increasing O_2 delivery. Investigation of the induction of EPO in response to hypoxia led to the identification of HIF-1α as a factor binding to an enhancer in the 3' region of this gene. In numerous publications, Semenza and colleagues further characterized HIF-1α as a bHLH-PAS transcription factor, by also determining its dimerization partner, HIF-1β, and demonstrating that the DNA binding activity of HIF-1α under hypoxia was a general

cellular response to decreased oxygen tension (Dengler et al., 2014). The discovery of a second oxygen-sensitive nuclear factor by independent research groups followed soon thereafter. HIF-2α shares high sequence homology with HIF-1α and functions in a similar manner. HIF-2α also dimerizes with HIF-1β upon hypoxic induction and stimulates the expression of a distinct set of target genes. Although identified via homology searches for additional bHLH-PAS and HIF-related proteins, the third known alpha subunit, HIF3-α, displays less similarity as well as distinct functional characteristics compared to HIF1-α and HIF-2α (Dengler et al., 2014). Interestingly HIF-3α exists as multiple splice variants, some of which inhibit HIF-1α and HIF-2α activity in a dominant-negative way (Majmundar et al., 2010).

Overall, the HIF family possesses a conserved protein domain structure. The three alpha isoforms as well as HIF-1β carry an amino-terminal bHLH domain that is necessary for DNA binding as well as PAS-A and PAS-B domains that are required for heterodimerization. Both the bHLH and PAS domains exhibit strong sequence and functional conservation among the HIFs. In fact, while the PAS domains between HIF-1α and HIF-2α exhibit approximately 70% identity, their bHLH domains share 85% identity with the basic region consisting of almost identical sequences. Comparatively, the bHLH and PAS domains of HIF-3α (common to most isoforms) share only 74% and 52-58% identity with HIF-1α and HIF-2α, respectively, which reveals the more divergent nature of this paralog (Dengler et al., 2014).

HIF-1α and HIF-2α also carry N- and C-terminal transactivation domains (N-TAD and C-TAD) that are required for activation of HIFs target genes. Oxygen-dependent degradation domains (ODD) within the alpha subunits confer oxygen-regulated turnover and overlap the N-TADs. The ODD is a highly conserved domain that controls the activity and stability of the alpha subunits, as it contains the key asparagine (N) and proline (P) residues targeted for hydroxylation in normoxic conditions. The presence of two distinct TADs confers the alpha subunits with both common and unique regulatory properties. Although the C-TAD exhibits the highest degree of sequence divergence, it acts to regulate the transactivation of target genes common to both HIF-1α and HIF-2α through coactivator recruitment. On the other hand, the N-TADs of HIF-1α and HIF-2α confer target gene selectivity between these two family members and this could be explained by the action of distinct transcriptional cofactors recognizing the N-TAD of HIF-1α versus HIF-2α (Dengler et al., 2014).

Under normoxic conditions, HIF-α subunits are continuously transcribed and translated. Sufficient oxygen tension however leads to their proteasomal degradation. In normoxia, a family of prolyl-4-hydroxylases (PHD1-4), most prominently PHD2, hydroxylate the oxygen-sensitive domain of alpha subunits. The PHDs require iron, α-ketoglutarate, ascorbate and molecular oxygen in order to modify conserved proline residues found in the ODDs of HIF-1α, HIF-2α. Proline hydroxylation allows binding of the von Hippel Lindau (VHL) E3 ubiquitin ligase complex, which poly-ubiquitinates the α-subunits then triggering their degradation by the proteasome.

In addition to prolyl hydroxylation, the HIF-1α and HIF-2α subunits are subject to hydroxylation of an asparagine residue in the transactivation domain (Asn-803 in human HIF-1α), which blocks interaction with the coactivator proteins CBP and P300. Asparagine hydroxylation and repression of HIF transcriptional activity is mediated by factor inhibiting HIF-1 (FIH-1), which is also an O_2^- and α-ketoglutarate-dependent dioxygenase with biochemical properties that are similar to those described above for the PHDs, although FIH-1 activity may be inhibited at higher O_2 levels than those required for inhibition of PHD activity (Samanta et al., 2017).

Of interest, experiments in which components of the electron transport chain in mitochondria have been inhibited evidenced the role of this organule in regulating PHD activity. In moderate hypoxia (1.5% O_2), mitochondria stimulate the production of cellular reactive oxygen species (ROS), which inhibit PHD activity and HIFα degradation. Hypoxia-induced oxidants were observed in the inner-membrane space of mitochondria as well as in the cytosol, where ROS could influence PHD activity. These findings support a model in which mitochondria sense O_2 deprivation and produce ROS to regulate PHD activity. The role of mitochondria in O_2 sensing may be restricted, however, to moderate hypoxia (1.5%). As O_2 levels decline further to anoxia (0% O_2), HIFα can be stabilized in the absence of functional mitochondria, suggesting that factors in addition to mitochondrial ROS antagonize PHD activity in more severe conditions of O_2 deprivation (Majmundar et al., 2010). O_2-sensing via hydroxylases and mitochondria define a core feature of HIF regulation. However, the list of additional cues, which modulate the HIF pathway, is growing. These factors range from microRNAs to oncogenic signals. One of the more interesting aspects of HIF regulation is rapresented by *sirtuins*, a stress-responsive family of nicotinamide adenine dinucleotide (NAD+)- dependent histone deacetylases. These enzymes represent sensors of the cellular redox state and modulate HIF activity highlighting the link between cellular stress and HIF responses. Among this family

members, Sirt1 regulates HIF-1α and HIF-2α through distinct machanism. Sirt1 forms a complex with HIF-2α, but not with HIF-1α, and deacetylates lysine residues in the protein thus enhancing its trascriptional activity. It was recently reported that Sirt1 can also interact with and deacetylates HIF-1α. In contrast to HIF-2α, HIF-1α trasnscriptional activity is repressed by acetylation. Moreover, overexpression studies indicate that HIF-2α can outcompete HIF-1α for binding to Sirt1. This observation underscore the complexity of the HIF response, and suggest that HIF-α subunits require dinstinct forms of regulation because they mediate non-overlapping biological effects (Majmundar et al., 2010).

It is believed that approximately 1-5% of the genome is transcriptionally regulated by hypoxia and many of these genes are known to be regulated by HIFs. So far, more than 200 target genes have been reported to be induced by the HIF complex. These genes are involved in many different biological processes, including erythropoiesis, angiogenesis, proliferation, energy metabolism or apoptosis. However, the gene expression pattern in response to the HIF activation is cell-specific. Therefore, the protective response by the HIF activation in one cell lineage may not be evident in other cell types (Gilany & Vafakhah, 2010).

3.1 HIFs-MEDIATED RESPONSES TO HYPOXIA

Erythropoiesis

A primary goal of increasing O_2 delivery systemically is to increase erythropoiesis. Erythropoietin is a glycoprotein hormone produced by the kidney that binds to receptors on erythroid progenitor cells in the bone marrow and stimulates their survival, proliferation, and differentiation. EPO levels increase in response to anemia or systemic hypoxia, leading to increased blood O_2-carrying capacity. HIF-1α was originally identified as a nuclear DNA-binding protein that was induced by hypoxia and bound to an HRE in the human EPO gene, which encodes erythropoietin. Subsequent studies have revealed that HIF-2α plays a critical role in regulating erythropoiesis by activating transcription of EPO and genes encoding proteins that are required for the absorption and delivery of iron to the bone marrow (Prabhakar & Semenza, 2015).

Angiogenesis

Chronic hypoxia induces angiogenesis. In general angiogenesis represents the process by which new blood vessels develop from existing vasculature; more specifically angiogenesis can be distinguished from other characteristic mechanisms of vessel growth. Vasculogenesis is the term which indicates the mechanism leading to the formation of vascular structures through the recruitment of bone marrow-derived endothelial progenitor cells to be incorporated into nascent vessels or to stimulate new vessel growth by releasing proangiogenic factors. Arteriogenesis is a process consisting of remodeling of existing blood vessels via recruitment of smooth muscle cells. Collateral vessel growth is a process consisting of the expansive growth of pre-existing collateral vessels following occlusion of supply vessels. Finally, tumors can also employ an additional mode of vessel formation through co-option of preexisting vessels or through formation of tumor vessels lined by tumor-derived cells or by endothelial cells with mutations in their chromosomes and derived from putative cancer stem cells (Cannito et al., 2014).

In any case, angiogenesis is a multistep process which involves endothelial cell (ECs) activation, increased blood vessel permeability, and local rearrangement of the basal membrane and extracellular matrix. Angiogenesis provides an efficient mechanism for the maintenance of an adequate blood flow to areas in which oxygen supply is insufficient. ECs and smooth muscle cells, by constituting the first-line cellular interface with blood, must necessarily have mechanisms to sense differences in the O_2 supply. This of course includes the already mentioned role of HIFs but also the O_2-sensitive NADPH oxidase, endothelial nitric oxide synthase (eNOS) and heme-oxygenases. In the list should also be included the complex I and III of mitochondrial electron transport chain, resulting in H_2O_2 generation, which is a well-known redox mediator able to stabilize functional HIF-α subunits (Cannito et al., 2014).

Accumulating evidence indicates that hypoxia can control the fates of vascular cells already during embryo development by stimulating the differentiation of various progenitors, including those of early mesoderm, into hemangioblasts and then into endothelial cells in the process of vasculogenesis. Moreover, HIF-ß may act during the first steps of vascular development, through paracrine release of vascular endothelial growth factor (VEGF) and angiopoietin-1 (Ang-1) by hematopoietic cells (Ramírez-Bergeron et al., 2006). However, hypoxia can also trigger differentiation of pulmonary artery endothelial cells into SMC-like (Zhu et al., 2006) cells and has been reported to promote in the adult bone marrow (BM) the differentiation of endothelial progenitor cells (EPCs) into

cells overexpressing the classic markers of mature vascular cells (Tillmanns et al., 2008). The literature data also indicate that hypoxia, via HIF-1α-dependent upregulation of stromal-derived factor 1 (SDF-1) by cells in hypoxic normal and tumor tissues, can recruit in these tissues various progenitors from bone marrow expressing SDF-1 receptor CXCR4, including circulating EPCs, pericyte progenitors and angio-competent $CD45^+$ myeloid cells (Fraisl et al., 2009; reviewed in Cannito et al., 2014).

Angiogenesis requires production and secretion of the so-called pro-angiogenic factors, such as vascular endothelial growth factor (VEGF), platelet-derived growth factor (PFGF), fibroblast growth factor (FGF), and interleukin-8. In keeping with the central role of HIFs in responses to chronic hypoxia, it has been shown that HIF-1α can directly activate the expression of genes involved in angiogenesis, including VEGF, VEGF receptor FLT-1, transforming growth factor-β3 (TGF-β3), angiopoietins and genes involved in matrix metabolism (Semenza, 2003).

The most notable and characteristic angiogenesis factor induced by HIF-1α is VEGF. It is a master pro-angiogenic cytokine that has been reported to sustain both physiological and pathological angiogenesis, whereas other members of the VEGF family, like for example placental growth factor (PLGF), may have a role only in pathological angiogenesis (Cannito et al., 2014). VEGF is essential for the proliferation and migration of vascular endothelial cells, thereby enabling the formation of new blood vessels (Gilany & Vafakhah, 2010). VEGF expression is unaffected in mice with endothelial cells-specific deletion of HIF-2α, implying that it is a HIF1α-specific target but these mice exhibit homeostatic defects in vessel integrity as well as impaired tumor angiogenesis (Skuli & Simon, 2009). These findings indicate that HIF-2α instructs endothelial cells to form more functional blood vessels suggesting it also may play a cell-autonomous role in this cell type (Majmundar et al., 2010). For istance Elvert et al. show that HIF-2α protein is highly expressed in brain capillary endothelial cells of mice during angiogenesis and is localized to endothelial cell nuclei were promote the expression of distinct genes (Elvert et al., 2003).

Metabolism

The erythropoietic and angiogenic responses to hypoxia do not increase O_2 delivery immediately, since it may take days to weeks to increase red blood cell counts or generate new blood vessels that are sufficient to improve tissue oxygenation. In contrast, responses

designed to reduce O_2 consumption occur over the course of hours to days. The principal metabolic response to hypoxia is a switch from oxidative to glycolytic metabolism. During hypoxia oxidative phosphorylation is arrested and glycolysis was stimulate with an increase in glucose consumption and lactate production. HIF-1α functions as a master regulator of glucose metabolism by activating the transcription of multiple targets including PDK1 gene, encoding pyruvate dehydrogenase kinase 1, which phosphorylates and inactivates the catalytic subunit of PDH and the LDHA gene, which encodes lactate dehydrogenase, the enzyme that converts pyruvate to lactate (Prabhakar & Semenza, 2015).

Hypoxia and HIFs have been implicated in promoting reverse flux through the TCA cycle and lipogenesis through activity of isocitrate dehydrogenase-1 (IDH1), an enzyme that promotes reductive carboxylation of glutamine to α-ketoglutarate and citrate. Renal cell lines lacking VHL exhibit constitutive reductive carboxylation of glutamine to α-ketoglutarate for de novo lipid biosynthesis at normal O_2 tensions, implicating the O_2-labile HIF subunits in regulation of this oxygen-sensitive pathway. An obvious role exists for HIF-mediated control of mammalian cell metabolism and acetyl-CoA production for lipogenesis under hypoxia. Cells may adjust to low O_2 tensions through changes in metabolism and methods of ATP production, but are also known to adaptively alter strategies for macromolecular biosynthesis, using metabolic intermediates to fuel nucleotide and lipid synthesis (Shay & Simon, 2012). HIF-1α activity was more recently shown to influence the pentose phosphate pathway or PPP (Zhao et al., 2010). The PPP converts glycolytic intermediates into ribose-5-phosphate (R5P), a substrate for nucleotide biosynthesis. HIF-1α, therefore, redirects the metabolism of glucose for use both as an energy source and as a building block for RNA and DNA synthesis, and these adaptations are likely important for facilitating cell growth and survival in hypoxic tumors (Majmundar et al., 2010).

Many of the metabolic genes described above are directly regulated by HIF-1α but not HIF-2α. It was demonstrated that HIF-2α stimulates the expression of genes encoding anti-oxidant enzymes, such as SOD2, in order to suppress aberrant ROS accumulation. in renal cancer has been demonstrated that HIF-2α plays a similar function: its depletion leads to reduced expression of genes with anti-oxidant functions, such as heme oxygenase 1 (HO-1) and others (reviewed in Majmundar et al., 2010). Although HIF-2α does not alter glucose utilization directly, it could function indirectly through regulation of PPARα and Pdk4 expression that inhibits the mitochondrial consumption of glucose-derived carbon

(Huang et al., 2002). In the setting of hypoxic stress, lipid metabolism is reprogrammed to suppress mitochondrial oxidation of lipid-derived carbon. Specifically, hypoxia stimulates lipid storage and inhibits lipid catabolism through β-oxidation. It was previously unclear if HIFs control these adaptations but a recent study implicates HIF2-α in the regulation of lipid metabolism (Rankin et al., 2009).

Inflammation

A reduction of blood flow at the inflammatory site and an increased metabolic demand from infiltrating immune cells and pathogens can eventually lead to the local depletion of O_2, resulting in hypoxic conditions. Hypoxia and associated HIFs involvement have been observed in tissue specimens from patients with inflammatory conditions and it is becoming increasingly clear that HIFs represent a common link between hypoxia and chronic inflammation through their function in macrophages (Majmundar et al., 2010). HIF-1α, in particular, is implicated in triggering the release of NF-kB-regulated inflammatory cytokines in stimulated macrophages. Along these lines, the existence of a complex feedback loop between HIF-1α stabilization and expression and the NF-kB pathway has been proposed. NF-kB activity in macrophage is required for HIF-1α stabilization and induction of HIF-1α target genes (Rius et al., 2008). Under hypoxia, the NF-kB activator IKK-β is stimulated and IkB phosphorylated. Subsequent IkB degradation liberates NF-kB from the cytoplasm, resulting in the transcription of downstream target genes, including those encoding for inflammatory cyotkines (Shay & Simon, 2012). In contrast, hypoxic induction of HIF-2α is not apparently dependent on NF-κB (Rius et al., 2008). In macrophages TH1 cytokines induce the activation of NF-κB pathway and the stabilization of HIF-1α while HIF-2α are usually stabilized in macrophages exposed to IL-4, a TH2 cytokine (Takeda et al., 2010). It should be noted, however, that available data on HIF-2α regulation in macrophages are somewhat conflicting. Conditional HIF-2α loss in myeloid cells decreases macrophage motility and invasion and reduces the severity of inflammation, with macrophages lacking HIF-1α exhibiting decreased motility, invasiveness, and bactericidal activity (Peyssonnaux et al., 2005). HIF-1α controls the metabolic shift from oxidative phosphorylation to glycolysis in activated macrophages and HIF-2α regulates the transcription of cytokines, such as IL-1β, IL-12, and TNFα, as well as genes involved in macrophage migration and chemotaxis. Indeed, a recent studies reviewed in Majmundar et al. (2010) confirms that HIF-1α and HIF-2α coordinate the behavior of different immune cells in a hypoxic inflammatory milieu.

Neutrophils, another major component of the innate immune response, are among the first cells recruited to inflammatory sites. Studies investigating HIF-1α specifically in neutrophils demonstrated that hypoxia-induced inhibition of neutrophil apoptosis is dependent on HIF-1α and that HIF-1α is required for phagocytes to fully exert their bactericidal activity (Peyssonnaux et al., 2005). Like HIF-1α, neutrophil HIF-2α contributes to hypoxia-induced inhibition of apoptosis. HIF-2α deficiency increases neutrophil apoptosis in vivo and ex vivo, leading to suppression of neutrophilic inflammation and inflammatory responses during acute lung injury (Thompson et al., 2014; reviewed in Lin & Simon, 2016).

Low O_2 tensions are also exhibited by regions of intense inflammation such as areas within solid tumors characterized by infiltration of myeloid-derived cells, better known as tumor-associated maxrophages or TAMs. VEGF is one of the chemokines recruiting TAMs in the regions of solid tumors and is a well-documented HIF-dependent hypoxia-induced target. Hypoxia appears to both regulate the expression of and play a role in modyfing chemoattractant receptors on the surface of these macrophages (Shay & Simon, 2012). In mouse models of hepatocellular carcinoma and colitis-associated colon carcinoma, mice lacking HIF-2α in their myeloid cells exhibit decreased recruitment of TAMs into tumor areas (Imtiyaz & Simon, 2010). This finding correlates with reduced tumor mitotic index, lower tumor grade, and a downward trend in the number and size of colitis-induced colon carcinomas. Recent work suggests that the absence of HIF-1α in macrophages has no effect on tumor spheroid infiltration, tumor cell proliferation or tumor invasiveness in vitro, but reduces cell death in tumor spheroid cultures (reviewed in Majmundar et al., 2010).

Cell-cycle

A fundamental physiological response to hypoxia is cell cycle arrest. HIF-1α is necessary for hypoxia-induced cell cycle arrest, and forced expression of HIF-1α is sufficient to induce G1-phase cell cycle arrest due to inhibition of Myc activity and direct interaction of HIF-1α with protein components of the pre-replication complex that blocks DNA replication (Prabhakar & Semenza, 2015). The cyclin-dependent kinase CDK2, which is active from late G1 through S phase and G2, binds to HIF-1α and triggers lysosomal degradation of the protein by chaperone-mediated autophagy, whereas CDK1, which is active from late G2 through M phase, binds to HIF-1α and protects it from lysosomal

degradation. Thus the cell cycle-specific regulation of HIF-1α by CDK1 and CDK2 allows HIF-1α to perform its role (with HIF-1β) as a transcription factor to mediate adaptive responses to hypoxia while preventing HIF-1α from performing its non-transcriptional role as an inhibitor of DNA replication. Coversely, HIF-2α has been shown to have an opposing effect to HIF-1α on c-Myc activity. Induction of HIF-2α results in increased of both stabilization of c-Myc complexes and transcription of its targets such as cyclin D2. In addition, HIF-2α has also been shown to regulate Oct4, a gene that can promote stem cell phenotypes and induce cyclin D1, hence promoting cell growth. More recently, HIF-2α was shown to engage mTORC1 activity, and promote cell proliferation through the regulation of the amino acid transporter, SLC7A5. It is thought that this are some of the mechanisms by which HIF2-α promotes hypoxic cell proliferation and tumourigenesis (Ortmann et al., 2014; Prabhakar & Semenza, 2015).

EMT

Epithelial to mesenchymal transition (EMT) is a key feature of invasive cells and can be characterized by the loss of epithelial cell-cell contact and the acquisition of mesenchymal features and motility. Results obtained in our laboratory revealed that different cancer cells (from human hepatoblastoma and pancreatic, colon and breast carcinoma) responded to hypoxia by classic EMT changes (fibroblastoid phenotype, SNAIL and β-catenin nuclear translocation and changes in E-cadherin) and by increased migration and invasiveness. Through the silencing of HIF-1α and the specific inhibition of mitochondrial generation of reactive oxygen species (ROS), we observed that early EMT-related events induced by hypoxia were dependent on transient intracellular increased generation of ROS whereas late migration and invasiveness were sustained by HIF-1α- and VEGF-dependent mechanisms (Cannito et al., 2008). Our results are in line with recent studies revealing that HIF-1α expression in renal cell carcinoma is sufficient to induce the loss of E-cadherin and an increase in invasion (reviewed in Kaelin, 2008). Moreover, in prostate cancer HIF-1α promotes SNAIL1 nuclear localization in a VEGF dependent manner. HIF-1α also induces lysyl oxidase (LOX), which is an extracellular matrix remodeling enzyme as well as an upstream regulator of SNAIL1 (Mak et al., 2010). Interestingly, LOX secreted by the primary tumor remodels distant premetastatic sites to recruit tumor and stromal cells (Erler et al., 2009). The hypoxic tumor microenvironment therefore promotes metastasis via the activation of multiple HIF-responsive genes that together regulate all stages of cancer

spread, including invasion, intravasation, and distant extravasation (Majmundar et al., 2010).

Tumorigenesis

Surprisingly, HIF-1α expression correlates with lower cancer stage or decreased patients mortality in certain cancers (i.e. neuroblastoma) while HIF2-α expression in these malignancies is a negative prognostic factor (Bertout et al., 2008). This difference between HIF-1α and HIF-2α expression suggests that HIF-α subunits may contribute differently towards tumorigenesis in certain cancers. Overexpression and knockdown studies of HIF-1α and HIF-2α in VHL-deficient renal cell carcinoma cell lines indicate that HIF-2α, but not HIF-1α, is necessary for tumor growth (Raval et al., 2005). A possibile explanation of this effect can be found in the different modulation of oncogenes and tumor suppressors. Microarray profiling revealed that compared to tumors expressing both HIFα isoforms, tumors exclusively expressing HIF-2α up-regulate MYC target genes, proliferate faster, and are relatively resistant to replication stress (reviewed in Majmundar et al., 2010). Another mechanism by which HIFs exert opposing effects on tumor behavior lies in the hypoxic regulation of the tumor suppressor protein p53. HIF-1α binds to p53, resulting in p53 stabilization and hypoxia-induced cell death. In contrast, recent experiments have shown that HIF-2α indirectly suppresses p53 activity and thereby promotes radioresistance and chemoresistance in tumor cells (reviewed in Majmundar et al., 2010). These findings indicate that certain cancers may differ in their tumor behavior and drug response according to the expression of HIF-1α and/or HIF-2α.

Other recent studies in non-VHL malignancies demonstrated that shRNA-mediated inhibition of HIF-2α, but not HIF-1α, in multiple human cancer cell lines reduces cell proliferation in vitro and subcutaneous xenograft growth in mice (Franovic et al., 2009). In vivo models of tumorigenesis suggest a more complex role for HIF-2α in cancer, assuming that different HIF-2α targets have varying activation thresholds-due to HRE sequence conservation, co-regulation by other transcription factors, composition of the transcription machinery, etc. - then according to the extent of HIF-2α stabilization, either tumor suppressive or tumor promoting effects may ensue (reviewed in Majmundar et al., 2010).

Recent studies indicates that other transcriptional regulators can complement the HIF response to O_2 deprivation. PHDs, for exemple, can play significant HIF-independent roles in cancer and suppress growth of xenograft tumors in a HIF-independent fashion (Chan et

al., 2009; reviewed in Majmundar et al., 2010). These observations emphasize that hypoxic adaptations are mediated by more than the HIF response.

4. LIVER HYPOXIA

The liver is the central metabolic organ responsible for maintaining blood glucose levels, ammonia metabolism, for biotransformation of xenobiotics and endogenous metabolic by-products of metabolism, as well as for bile synthesis. All these processes require that a number of pathways and enzyme reactions are running in parallel in the most efficient manner. To achieve this, the liver parenchyma displays a functional organization known as metabolic zonation. On the histological level, the lobule represents the smallest unit with a hexagonal shape and the central vein in the middle. The corners of the hexagon are formed by so called portal triads consisting of a branch from the portal vein, and a branch from the hepatic artery as well as a bile duct. In the lobule, the hepatocytes are connected with each other and are visible as cords. In the cords the hepatocyte membranes are interconnected and face blood channels called sinusoids at either side. The sinusoids are wrapped with lines of fenestrated endothelial cells and are also populated with Kupffer cells. Importantly, there is a small space between the endothelial cell lining and the apical membrane of the hepatocytes called the Space of Disse; it is involved in lymph draining and provides a resident niche for hepatic stellate cells (HSCs) which store fat and vitamin A (Sasse et al., 1992). In addition to the lobule, the *acinus* can be visualized by connecting two portal triads with a line from which it extends into the direction of the two adjacent central veins. The acinus represents the functional zone in terms of blood flow and is composed by three zones, one around the portal triads, i.e. (the periportal zone), a second intermediary zone (zone 2) and the third around the central vein (zone 3, the centrilobular zone) (reviewed in Kietzmann, 2017). The key enzymes of various pathways and thus the metabolic capacities are found to be preferentially located in one of these zones, a pattern that became commonly known as metabolic zonation (originally described in Katz & Jungermann, 1976). The zonation allows that opposing pathways are spatially separated which prevents competition for a common substrate and futile cycles, that complementing pathways can be linked, and substrate demanding activities can be carried out at sites with the best substrate provision. Metabolic zonation has been shown for carbohydrate, amino acid, lipid, ammonia, and xenobiotic metabolisms (Jungermann & Katz, 1989). Moreover, the localization and functional activities of non-parenchymal cells also are zonated. Zonation

is rather dynamic and not static since most gene expression patterns and consequently enzyme distributions change in response to nutrition, drugs, hormones, and other blood borne factors (Kietzmann, 2017).

Gradients of morphogens (e.g. Wnt, hedgehog, hormones) or growth factors such as HGF and other factors such as oxygen act in concert, in order to restrict gene expression to differentiated hepatocytes located in specific zones of the liver acinus. The oxygen is of particular importance and ranges from about 60–65mm Hg (84–91 μmol/L) in the periportal blood to about 30–35mm Hg (42–49 μmol/L) in the perivenous blood. Accordingly, the intracellular pO_2 is about 15mm Hg lower, i.e. 45– 50mm Hg in periportal cells and 15–20mm Hg in perivenous cells (Kietzmann, 2017). This goes in line with differences in the number and structure of mitochondria as well oxidative capacities in periportal and perivenous zones.

Evidence for the role of oxygen as a modulator of zonation came from the observation that in livers of mice transgenic for the human erythropoietin (EPO) gene, human EPO mRNA was detected only in the less aerobic perivenous hepatocytes (Koury et al., 1991).

In line with the oxygen gradient in liver, all HIF-α subunits are found with higher levels in the less aerobic perivenous zone. Indeed, the first rim of perivenular hepatocytes has been reported to positively stain for HIF-1α (i.e., in the nuclei) as well as for vascular endothelial growth factor A (VEGF-A), a typical HIF- related target gene (Cannito et al., 2014). The importance of HIF- transcription factors for structural maintenance of the liver is exemplified in studies from mice with hepatocyte-specific HIF-1α deficiency. Those mice displayed an extension of hepatic lobules, an enhanced lobular oxygen consumption and an increased content of mtDNA (Tsukada et al., 2013). Further, HIFs regulate major metabolic liver functions, and in particular studies in which either ARNT or the two HIFα subunits were deleted supported this concept. Liver-specific ablation of the common beta HIF subunit (ARNT) in mice increased fed insulin levels, gluconeogenesis, lipogenesis, and decreased ketone bodies (X. L. Wang et al., 2009). Deletion of HIF-1α has been shown to impair gluconeogenesis during liver regeneration whereas HIF-2α is crucial for hepatic insulin signaling and, accordingly, its constitutive activation in mouse liver resulted in development of severe hepatic steatosis associated with impaired fatty acid beta-oxidation, decreased lipogenic gene expression, and increased lipid storage capacity (Kietzmann, 2017).

5. HIFs & CHRONIC LIVER DISEASES

Chronic liver diseases (CLDs) are a significant global public health issue. CLDs caused by viral infection, alcohol abuse, or obesity-associated metabolic disorder can develop into end-stage liver cirrhosis and liver cancer. It is estimated that in 2013 alone, there were 792.000 new cases of liver cancer and 818.000 deaths globally, with 86% occurring in developing countries and 14% occurring in developed country (Ju et al., 2016).

Increased expression of HIF-1α and HIF-2α has been reported in many liver diseases, including nonalcoholic fatty liver disease (NAFLD), alcoholic liver disease (ALD) and hepatocellular carcinoma (HCC). A common feature of these liver diseases is tissue hypoxia due to an imbalance of metabolic demand and supply. Oxygen consumption by hepatocytes and infiltrating inflammatory leukocytes is dramatically increased and in the meantime, oxygen supply is reduced due to vascular dysfunction, thrombosis, or fibrosis. Aside from hypoxia, other conditions associated with liver disease can also stabilize HIFs. For example, hepatic endotoxin levels are increased in NAFLD and ALD, and lipopolysaccharide (LPS) has been reported to stabilize HIFs. LPS-induced HIF activation is dependent on toll-like receptor (TLR)-4 and mediated through NF-κB and MAPK pathways (Peyssonnaux et al., 2007). During inflammation, the TCA cycle intermediate succinate is increased, and it functions as a HIF activator (Tannahill et al., 2013). Reactive oxygen species (ROS) have also been shown to stabilize HIFs, and increased production of ROS is a common phenomenon in liver pathological conditions (Guzy et al., 2006; Novo et al., 2012). Furthermore, evidence suggests that hepatitis B and C viruses directly stabilize HIFs under normoxic conditions (Ju et al., 2016).

5.1 HIFs and NAFLD

NAFLD consists of a spectrum of disorders from simple fat accumulation in the liver (steatosis) to non-alcoholic steatohepatitis (NASH). Steatosis is defined as deposition of triglycerides exceeding 55 mg per gram of liver or the presence of triglycerides droplets in more than 5% of hepatocytes (Szczepaniak et al., 2005). Although steatosis is benign initially, it represents a key step in the development of hepatitis with hepatocyte damage, infiltration of inflammatory leukocytes, and collagen deposition (fibrosis).

In approximately 10– 30% of individuals with nonalcoholic steatohepatitis, the disease progresses into liver cirrhosis within 10 years, with cirrhotic but also non-cirrhotic patients reported to develop hepatocellular carcinoma (HCC) (Argo et al., 2009).

Multiple lines of evidence suggest that hypoxia may play a role in hepatic lipid accumulation promoting hepatic steatosis. In patients and animals with NAFLD, HIFs stabilization has been observed and it is reported that hypoxia induces genes involved in lipid synthesis, storage, and uptake (Furuta et al., 2008).

Mice with hepatocyte-specific deletion of VHL, resulting in the overexpression of both HIF-1α and HIF-2α, also exhibit increased hepatic lipid accumulation as compared with WT mice. Between the two isoforms, HIF-2α seems to play a more important role in regulating hepatic lipid metabolism (Rankin et al., 2009). In the PHD2/PHD3 double knockout mice, it has been demonstrated that HIF-2α, but not HIF-1α, is associated with steatosis (Minamishima et al., 2009). Similarly, in the VHL-deficient mice, deletion of HIF-2α, but not HIF-1α, attenuates hepatic steatosis, suggesting that HIF-2α is the main promoter of steatosis. Although the direct molecular targets of HIF-2α are not clear, published data indicate that transgenic mice characterized by HIF-2α overexpression exhibit increased expression of lipogenic genes (SREBP1c and FAS), decreased expression of genes involved in fatty acid oxidation (PPARα and carnitine palmitoyl-CoA transferase-1, Cpt1) and up- regulation of the lipid droplet surface protein ADFP (Rankin et al., 2009). More detailed molecular studies are necessary to elucidate the mechanism of HIF-mediated regulation of lipid metabolism and therefore in the pathogenesis of NAFLD.

5.2 HIFs and LIVER FIBROSIS

Chronic liver injury and inflammation caused by viral hepatitis (HBV or HCV) infections, alcohol abuse, NAFLD, and biliary obstruction often results in tissue fibrosis. Fibrosis is part of a normal wound-healing response to tissue injury. However, repeated injury causes chronic inflammation, matrix deposition, and angiogenesis, which leads to progressive fibrosis. In some patients, liver fibrosis progresses to cirrhosis, in which hepatocytes are replaced by scar tissue with excess fibrillary collagen produced by hepatic stellate cells. Liver fibrosis causes an increased resistance to blood flow, which together with sinusoidal obstruction can cause tissue hypoxia (Ju et al., 2016). Interestingly, hypoxic areas colocalize with those of fibrosis, suggesting a link between hypoxia and fibrogenesis

(Corpechot et al., 2002; Valfrè di Bonzo et al., 2009). Moreover, HIFs stabilization has been reported in experimental models of liver fibrosis. Experiments performed in HIF-1α liver-conditional knock-out mice in a model of biliary fibrosis indicated that hypoxia and HIF-1α recruitment preceded fibrosis and that hepatocyte-specific silencing of HIF-1α resulted in a reduction of collagen synthesis and α-smooth muscle actin (α-SMA) staining, (i.e., two markers of liver fibrosis), as well as of selected pro-fibrogenic mediators including plasminogen activator inhibitor-1 (PAI-1) and platelet-derived growth factor (PDGF-A/B) (Moon et al., 2009).

Another report showed that hepatocyte-specific HIF-1α–deficient mice fed with ethanol concomitantly with CCl_4 treatment, resulted in the amplification of CCl_4-induced hepatic fibrosis. Hepatocyte-specific HIF-1α deficiency prevented the ethanol-induced increase in CCl_4-induced fibrosis compared with control mice, indicating an important role for hepatocyte HIF-1α in the development of liver fibrosis in this model. However, when mice were treated with CCl_4 alone without ethanol, HIF-1α deficiency in hepatocytes did not reduce liver fibrosis, which may indicate that hypoxia resulting from ethanol metabolism may enhance HIF-1α activation in hepatocytes after CCl_4 treatment (Roychowdhury et al., 2014). Data in support of a role for HIFs in liver fibrosis have been provided in other models of liver fibrosis: after 5 weeks of diethylnitrosamine (DEN) administration pronounced collagen septa can be observed, and progression to cirrhosis is observed by 8 weeks. In DEN-treated mice, VEGF isoforms were increased with increasing time of treatment and proposed to be correlated with an increase in tissue hypoxia (Corpechot et al., 2002b). Studies by Qu et al. (Qu et al., 2011) found that HIF-2α in hepatocytes may also be important for progression of fibrosis. In these studies, authors have performed their experiments using simultaneous, hepatocyte-specific VHL and HIF-1α or HIF-2α mouse mutants. In these mice hepatocyte specific disruption of VHL (and then with an increased expression of both HIF-1α and HIF-2α) resulted in steatosis as well as in increased inflammation and fibrosis. All these events were prevented when simultaneous deletion of HIF-2α, but not HIF-1α, was carried out. Furthermore, VHL-disrupted mice fed shortly with an ethanol diet produced an increase in fibrosis. This increase was completely prevented in hepatocyte-specific VHL- HIF-2α double knockout mice, but not in hepatocyte-specific VHL- HIF-1α double knockout mice. Together, these data indicated that HIF-2α activation in hepatocytes may be relevant for the development of liver fibrosis in this model (Qu et al., 2011).

In a study by Scott et al. (Scott et al., 2015) HIF-1β floxed mice were crossed with mice that express Cre recombinase under control of the albumin promoter to generate hepatocyte-specific HIF-1β knockout mice. Mice with and without deletion of HIF-1β in hepatocytes were treated with the hepatotoxin thioacetamide to induce liver fibrosis. Although the pattern of fibrosis was similar in both deleted and undeleted mice, hepatocyte-specific HIF-1β knockout mice showed a decrease in macrophage infiltration as well as a decrease in the mRNA expression of profibrotic genes, including TGF-β1 and TGF- β2, collagen type 1 and 5, and tissue inhibitors of metalloproteinases 1 and 5 (TIMP1 and 5). These additional observations once again suggest that HIFs may contribute to fibrosis development.

In a series of in vitro experiments, Copple and colleagues (2010) reported that production of pro-fibrogenic mediators was induced by culturing mouse hepatocytes in 1% oxygen. Using a siRNA approach, the authors demonstrated that the production of pro-fibrogenic mediators was completely prevented in ARNT-null cells, but only partially prevented in HIF1-α-null cells, suggesting that other HIF isoforms (particularly HIF-2α) may play a role. The same laboratories demonstrated that HIF-1α and HIF-2α are activated in hypoxic hepatocytes and regulate the expression of PAI-1 and VEGF as well as the activation of TGF-β1, which could promote the development of liver fibrosis (Copple, 2010; Copple et al., 2009).

It should be noted that pro-fibrogenic effects of HIFs have been reported also in other hepatic cell populations, including Kupffer cells, the resident liver macrophages, that are known to contribute to the pathogenesis of fibrosis by producing growth factors that stimulate HSC proliferation and collagen production. Recent studies demonstrated that HIF-1α was activated in scar-associated macrophages in both mice and humans with liver fibrosis (Copple et al., 2012). In another studies, Copple et al. (2009) showed that Kupffer cells from non-diseased livers exposed to hypoxia in vitro activated HIF-1α but not HIF-2α, suggesting that HIF-1α signaling may predominate in Kupffer cells. Takeda et al. (Takeda et al., 2010) showed that HIF-1α is up-regulated and HIF-2α is down-regulated in classically activated macrophages (i.e., M1 macrophages), whereas HIF-2α is up-regulated and HIF-1α down-regulated in alternatively activated macrophages (i.e., M2 macrophages). It is possible that Kupffer cells in non-diseased livers are skewed toward a classically activated phenotype, which may explain the lack of HIF-2α activation in these cells. In diseased livers, hepatic macrophages with activated HIF-1α or HIF-2α have been described, indicating that both transcription factors can be activated in hepatic

macrophages in vivo after injury. In mice subjected to BDL, knocking out HIF-1β selectively in macrophages did not affect liver injury or hepatic inflammation but reduce liver fibrosis as measured by type I collagen and α-SMA mRNA and protein levels (Copple et al., 2012). Similar results were observed in mice in which HIF-1α was knocked out in macrophages, suggesting that HIF-1α in macrophages is profibrotic (Copple et al., 2012). In this connection, it should be noted that very few data exist concerning the role of HIF-2α in macrophages.

Exposure to hypoxic conditions of cultured-primary mouse HSCs results in the activation of both HIF-1α and HIF-2α and increased the expression of several genes that could potentially contribute to fibrosis development (Copple et al., 2011). This is the case of hypoxia-dependent increased expression of two genes involved in collagen metabolism such as prolyl-4-hypdroxylase A1 and prolyl-4-hydroxylase A2, which are key enzymes that contribute to the formation of stable collagen triple helices (Copple et al., 2011). Literature data have proposed that angiogenesis may represent a critical factor in the development of liver fibrosis and, as expected, hypoxia also increased expression of genes involved in angiogenesis (i.e. VEGF, angiopoietin-like-4, placental growth factor, and macrophage-migration inhibitory factor) (Copple et al., 2011). However, the same cells are also sensitive targets of VEGF that has been reported to stimulate HSC/MFs proliferation and synthesis of ECM components as well as to stimulate oriented migration of these cells (Novo et al., 2012). Moreover, hypoxia itself is able to induce oriented migration of either HSC/MFs or MF-like mesenchymal stem cells from bone marrow by eliciting a biphasic mechanism: i) an early phase of migration switched on by ROS released by mitochondria and requiring redox-dependent activation of Ras/ERK and c-Jun-NH2-terminal kinase isoforms (JNKs); ii) a delayed and sustained phase of migration depending on HIF-1α-mediated, ROS stabilized, up-regulation of VEGF expression, resulting in the subsequent chemotactic action of extracellularly released VEGF. Relationships between hypoxia and ROS generation outlined in HSC/MFs have a counterpart in vivo in liver specimens from HCV cirrhotic patients, as documented by positive nuclear staining for HIF-2α and cytoplasmic staining for heme-oxygenase 1 (HO-1) in hepatocytes of regenerative nodules and in α-SMA-positive MFs (Novo et al., 2012). In this scenario it is correct to cite recent studies that have conversely shown that VEGF may be also critical for reversal of fibrosis; in particular, this is based on reported putative anti-fibrotic effects of VEGF which are most likely due to actions exerted by VEGF on sinusoidal endothelial cells rather than on

activated HSCs, then suggesting that the relevance of VEGF and angiogenesis may be more complex of what originally envisaged (Yang et al., 2014).

Hypoxia increases also the expression of the chemokine receptors CCR1 and CCR5 that are able to stimulates HSC migration (Copple et al., 2011). In support of a role for these receptors in the development of fibrosis, it was shown that Ccr1 and Ccr5 knockout mice have reduced liver fibrosis (Seki et al., 2009). Two other receptors of interest are increased by hypoxia in HSCs: interleukin-13 receptor A1 and adrenergic receptor A2b, which are activated by interleukin-13 and catecholamines, respectively (Copple et al., 2011). In vitro, activation of these receptors on HSCs stimulates collagen production and in vivo both these pathways have been shown to play an important role in liver fibrosis development (Weng et al., 2009). Although up-regulation of some of the genes here described required HIF-1α, the increased expression of others was HIF-1α - independent, indirectly suggesting a possible role for HIF-2α or potentially other hypoxia-modulated transcription factors. It has been proposed that since up-regulation of many of these genes by hypoxia only occurs in culture-activated HSCs, the process of HSC activation in culture may in some way increase the sensitivity of HSCs to HIF-mediated gene expression changes. The mechanism by which this may occur is not known although it may result from changes in histone modifications or DNA methylation that modify access of HIFs to the promoters of certain HIF-regulated genes in activated HSCs (Roth & Copple, 2015).

In addition to the genes already described, hypoxia also increased expression of the Jumonji domain-containing proteins Jmjd6, that regulates mRNA splicing, and Jmjd1a, a histone demethylase (Webby et al., 2009). Up-regulation of Jmjd1a by HIFs could increase expression of a number of genes by modifying histone methylation, whereas up-regulation of Jmjd6 could promote the formation of alternatively spliced mRNAs that encode for proteins with modified functions resulting in fibrosis promotion (Yamane et al., 2006).

Although numerous genes were increased in hypoxic HSCs, surprisingly few were decreased by hypoxia. It has been shown that hypoxia is able to rapidly inhibit c-met expression in hepatocytes and also to down-regulate synthesis and release by HSCs of hepatocyte growth factor (HGF), the most potent mitogen for parenchymal cells (Corpechot et al., 2002). Another gene of particular interest that is decreased by hypoxia is the collagen receptor α1-integrin. It has been demonstrated that the collagen production by fibroblasts from α1-integrin knockout mice is enhanced, suggesting a role for this receptor

in feedback inhibition of collagen production (Gardner et al., 1999). The importance of the decrease in α1-integrin in hypoxic HSCs, however, remains to be determined.

5.3 HIFs and VIRAL HEPATITIS

Hepatitis C virus (HCV) is a single-stranded RNA virus that infects and replicates in hepatocytes. HCV infection affects 180 million people worldwide with higher prevalence in Japan, Europe, and the US than other regions. Hepatitis B virus (HBV) is a member of the hepadnavirus family (Ju et al., 2016). HBV infection affects 350–400 million people, accounting for 5% of the world population. The majority of infected people live in Asia (75 %), whereas the prevalence is now very low in Western countries (<1 %) (Ju et al., 2016). Viral hepatitis often leads to serious end-stage liver diseases such as cirrhosis and HCC, with patients often requiring liver transplantation. Therefore, it remains important to identify new therapeutic targets in order to develop strategies to ameliorate liver injury caused by viral hepatitis and to slow down disease progression.

HIF-1α and HIF-2α stabilization has been observed in HBV- or HCV-infected cells and in liver biopsies from patients with chronic viral hepatitis. It has been demonstrated that the hepatitis B viral X protein (HBx), which is indispensable for viral replication, can directly interact with the bHLH/PAS domain of HIFα subunit, thereby decreasing its binding with pVHL and preventing degradation (Moon et al., 2003). Moreover, HBx is reported to activate MAPK pathway and in turn activate HIF-1α. It is also implicated in promoting angiogenesis by inducing transcriptional activation of angiogenic factors such as VEGF (Yoo et al., 2003).

Hepatitis C infection may interact with the HIF-1α pathway by way of multiple mechanisms. Transient HCV infection in Huh7 cells was associated with HIF1α stabilization. This stabilization appeared to be dependent on multiple kinase and transcriptional pathways, as functional ERK and PI3K inhibition were able to prevent HIF1α protein accumulation/stabilization, similar to what reported in conditions leading to Stat3 and NF-kB inhibition (Nasimuzzaman et al., 2007). HIF-1α stabilization by HCV, accompanied by production of functional VEGF, was shown to be insensitive to antioxidant treatment and dependent on derangement of mitochondrial respiration in HCV-infected cells (Ripoli et al., 2010).

HIFs may also promote viral transmission and replication in hepatocytes through modifying cell permeability and energy metabolism . It has been reported that HIF-induced VEGF production may depolarize hepatocytes and facilitate viral entry. HIF stabilization can also enhance cell survival, which indirectly promotes viral infection (Mee et al., 2009). Moreover, HIF-1α stabilization has been also reported to modulate tight junction proteins and promote migration of HCV-infected cells (Wilson et al., 2012). Although some experimental data support a role for HIFs in viral replication and cellular responses to viral infection, the transcriptional targets involved remain to be elucidated.

5.4 HIFs and HEPATOCELLULAR CARCINOMA (HCC)

HCC is one of the most common cancers worldwide with more than half a million new cases occurring each year. Because of its poor prognosis and lack of treatment options, HCC ranks as the fifth most common cause of death in men and the ninth in women. The majority of the HCC cases are associated with chronic hepatitis B and C infection occurring in less developed countries of East Asia and sub-Saharan Africa. However, in the past 20 years, the incidence of HCC in developed countries has increased significantly (Ju et al., 2016). Clinical observations reveal that liver cirrhosis is present in nearly 80% of patients with HCC, representing the most important risk factor for HCC development. Current treatment options for HCC are very limited. A small number of patients (15%) with early stage of the disease may be cured by liver resection or transplantation. However, in about half of the patients, tumor recurrence and metastases occur post-resection and it is then critical to better understand the pathogenesis of HCC in order to develop novel therapeutic strategies (Ju et al., 2016).

Hypoxia appears to induce a series of adaptive “pro-survival” changes in the tumor, which include a shift from aerobic to anaerobic metabolism, an increase in erythropoietin to promote rise in hemoglobin, and an increase in growth factors leading to angiogenesis. Furthermore, hypoxia has been associated with resistance to chemotherapy and radiotherapy, and is closely related to poor clinical outcome. Both HIF-1α and HIF-2α have been observed to be expressed at higher levels in HCC tissues than those in non-tumor-surrounding tissues (S. Li et al., 2011). The high expression levels of HIF-1α or HIF-2α have been correlated with worse tumor grade, venous invasion, intrahepatic metastasis, and capsule infiltration (Bangoura et al., 2007). HIF-1α and HIF-2α

stabilization is a poor prognostic marker, as it is associated with shorter disease free period and lower overall survival rate (Xiang et al., 2012). Multiple factors contribute to the stabilization of HIFs during HCC development. Angiogenesis, stimulated by hypoxia, is essential for cancer progression. HIF-1α and VEGF have been found to be expressed at higher levels in dysplastic nodules and implicated in malignant transformation (K. Nakamura et al., 2007). It has been shown that antiangiogenic therapy is effective in the treatment of HCC. Currently, the multikinase inhibitor *sorafenib* is still the only approved drug for patients with advanced HCC and it has been demonstrated that the mechanisms that account for the antiangiogenic efficiency of this drug are associated with its inhibitory effect on the expression of HIF-1α and VEGF proteins, leading to a decrease in vascularization of HCC (Luo et al., 2014). In addition to VEGF, HIF-1α also induces the expression of other angiogenic growth factors such as stromal derived factor-1 (SDF-1), angiopoietin 2 (ANGPT-2), placental growth factor (PGF), platelet-derived growth factor-B (PDGF-B), and stem cell factor (SCF) (Kim et al., 2010).

Cell proliferation and survival were sustained by HIF-1α inducing other growth factors such as TGFα and IGF-2. Moreover, HIF-1α directly binds to the promoter of Forkhead boxM1(FoxM1), a transcription factor that promotes proliferation in HCC cells and their resistance to apoptosis (Xia et al., 2012).

Another essential process of cancer biology promoted by HIF-1 is the activation of glucose aerobic metabolism, which allows tumors to survive under hypoxia. Metabolic re-programming from oxidative phosphorylation to aerobic glycolysis, termed the *Warburg effect*, is a common characteristic and survival mechanism of cancer cells. HIF-1α activates key enzymes that contribute to the Warburg effect, such as the glucose transporter 1 (GLUT1), which mediates cellular glucose uptake, as well as hexokinase (HK) and lactate dehydrogenase A (LDHA), which convert pyruvate to lactate. Lactate so produced can be removed from the cell through the action of monocarboxylate transporter 4 (MCT4), regulated by HIF-1α, while pyruvate dehydrogenase kinase 1 (PDK1) and MAX interactor 1 (MXI1) can block the flow of pyruvate into the mitochondria (Luo et al., 2014).

Tumor metastasis is the primary factor of poor prognosis in several cancers. To promote invasion and metastasis, HIFs can induce epithelial-mesenchymal transition by stimulating the transcription of several genes that are E-cadherin repressors, such as Snail, Twist1, transcription factor 3 (TCF3), Zfhx1a, and Zfhx1b. As already mentioned and in line with literature data, previous study from our laboratory reported that moderate hypoxic

conditions can trigger EMT in different human cancer cells, resulting in increased invasiveness. Hypoxia-dependent changes occur through a biphasic mechanism: i) an early stage, ROS-dependent inhibition of glycogen synthase kinase-3β (GSK-3β), followed by early Snail translocation and down-regulation of E-cadherin; ii) a later stage consisting in Wnt/β-catenin signaling activation and involvement of VEGF. These findings suggest that early redox mechanisms can turn-on the switch for hypoxia-dependent acquisition of EMT phenotype whereas increased invasiveness is sustained by HIF-1α-dependent release of VEGF (Cannito et al., 2008). In addition, HIF-1α upregulates expression of matrix metalloproteinases associated with the degradation of extracellular matrix (ECM) and therefore contributing to invasiveness of tumor cells. Along these lines, it has been reported that HIF-1α silencing by specific siRNAs can reduce MMP2 and MMP9 expression, thereby inhibiting migration and invasion (Okazaki et al., 2012).

The role of HIF-2α in HCC is less clear, and there have been conflicting reports. Recent evidence supports a possible tumor suppressor role for HIF-2α in HCC (Sun et al., 2013). However, several studies show that HIF-2α expression correlates with the progression of several cancers and several data indicate that HIF-2α has a greater oncogenic capacity than HIF-1α and that its overexpression correlates with poor patient outcome in colorectal carcinoma, melanoma, ovarian cancer and hepatocellular carcinoma, possibly by promoting Myc activity, and radio- and chemo-resistance through indirect suppression of p53 activity. In this connection, HIF-2α may act through up-regulation of KLF4, Sox2 and Octamer-4, to favor de-differentiation and confirmation of stem-cell properties of putative cancer stem cells (reviewed in: Keith et al., 2011).

The poor outcome of patients with HCC is related to the late detection of the cancer, with the majority of patients diagnosed at an advanced stage of disease. A major problem with HCC detection and surveillance is the lack of reliable biomarkers. Alpha-fetoprotein (AFP) is the most widely used serum marker for HCC diagnosis and surveillance; however, not all HCCs secrete AFP. Furthermore, AFP may be elevated in patients with chronic liver disease in the absence of HCC, making this biomarker inadequate for surveillance tests. From 1990's, especially in Japan, new biomarkers for HCC diagnosis and surveillance have been explored. Among these, des-γ carboxy-prothrombin, an abnormal prothrombin protein, has been considered (Li et al., 2014), but results obtained now indicate that its sensitivity is highly dependent on tumour size (Nakamura et al., 2006). The clinical utility of lens culinaris agglutinin-reactive fraction of AFP-L3 in early prediction of HCC development in patients with chronic HBV or HCV infection was also recently evaluated

(Schütte et al., 2015). It was shown that several factors (gender, age, race, and presence of more advanced liver disease) are independent predictors of increased levels of this biomarker, which also lacks in sensitivity, specificity, and predictive values required for routine HCC surveillance (Sterling et al., 2012). Another biomarker that has been developed in recent years is Osteopontin, a molecule expressed by transformed malignant cells, also evaluated for colon and pancreatic cancer (Wan et al., 2014). The majority of the studies analyzing osteopontin for the diagnosis of HCC were retrospective and included a range of 30 to 179 patients with HCC. Further validation studies are needed to use this marker in daily clinical routine (Wan et al., 2014).

On the basis of the above considerations, a reliable biomarker in detecting early HCC still represents a crucial unmet need. In recent years relevant emphasis has been ascribed to innate or natural immunity, which acts as the first line of defense, and also as the link between acquired immunity and immunological memory. Poly-reactive natural auto-antibodies [immunoglobulins M (IgM)] can bind, with low affinity and high avidity, different markers that are expressed during cancer growth (Vollmers et al., 2009). The presence of IgM-linked immune-complexes with diagnostic value has been found recently in different human tumors, including colon and prostate cancer, and also in other pathologic conditions. For liver disease, the diagnostic value of squamous cell carcinoma antigen-IgM (SCCA-IgM) immune-complex in serum has been demonstrated in several studies (Martini et al., 2015).

6. SERPIN-B3

SCCA belongs to the clade B subset of the Ovalbumin-serpin family. Serpins are serine protease-inhibitor and in mammals perform many important functions, especially in digestion, blood clotting, and the complement system. Serpins function *via* an exposed reactive site loop (RSL) of about 20 amino acids and inhibit irreversibly proteinases through a suicide substrate inhibition mechanism. The protease cleaves the serpin reaction site (RSL) leading to the formation of a covalent bond between the two molecules and an allosteric alteration in the serpin structure itself, an event that allows subsequent degradation of the protease (Gettins, 2002; Khan et al., 2011).

SCCA1 (SERPINB3) and its isoform SCCA2 (SERPINB4) are over-expressed in squamous cell carcinoma (SCC) of the uterine cervix, lung, head and neck, rectal colon,

pancreatic and liver tumors. The isoform that has been better evaluated in literature is SERPINB3, which has showed functional connection with tumorigenesis. This isoform was found to prevent cell death through its binding to complex 1 of the mitochondrial respiratory chain or via suppression of c-JUN, as a response to different types of stress, such as UV, radiation, chemotherapy, tumour necrosis factor-alpha and natural killer cells. Moreover, its inflammatory and pro-tumorigenic role has been revealed demonstrating its ability to enhance IL-6 effects through NF-kB pathway in response to Rat Sarcoma Viral Oncoprotein stimuli (Martini et al., 2015).

SERPINB3 is undetectable in normal hepatocytes, but their expression progressively increases from chronic liver disease to dysplastic nodules and HCC, suggesting a role in hepatocarcinogenesis. In HCC, high expression of SERPINB3 is significantly associated with early tumour recurrence, and shows a better prognostic significance than other clinical and histological variables. These important clinical findings were confirmed at the molecular level: SCCA/SERPINB3 expression in liver tumors has been correlated with liver regeneration activity; moreover, increased proliferation was also documented in hepatoma cell lines overexpressing SERPINB3 and in a mouse model transgenic for this serpin. Recent data indicate that SERPINB3 is highly expressed in the hepatic progenitor cell compartment of both fetal and adult livers (Martini et al., 2015).

In the study of Quarta et al, it was demonstrated that SERPINB3, acting also as a soluble factor, was able to induce EMT-related changes and increased invasiveness in HepG2 and MDCK (Madin Darby Canine Kidney cells) (Quarta et al., 2010).

Concerning SERPINB3 and hypoxia, the results obtained previously in our laboratory provide evidence for the first time that SERPINB3 expression is significantly up-regulated by hypoxia through HIF-2α-dependent mechanisms in human liver cancer cells. Data from ChIP assays revealed that HIF-2α (not HIF-1α) binds to SERPINB3 promoter very early after exposure to hypoxia and that this is paralleled by the binding of RNApol II, indicating that transcription of SERPINB3 gene is active following exposure to hypoxia. It has also been proven that the HIF-2α-dependent up-regulation of SERPINB3 requires generation of ROS and redox-mediated signaling. Indeed the rise of both ROS and SERPINB3 mRNA levels was abolished by pre-treating cells with rotenone, a pharmacological inhibitor of mitochondrial electron transport chain known to block hypoxia-induced mitochondrial release of ROS and HIF-1α and HIF-2α stabilization. The hypoxia-dependent and ROS-related up-regulation of SERPINB3 involves activation and phosphorylation of Ras/ERK

signaling cascade, since the pre-treatment of hypoxic HepG2 cells with pharmacological inhibitor of MEK (PD 98059) completely abolished SERPINB3 expression. By performing an analysis on liver specimens obtained from a well characterized cohort of 67 HCC patients the existence of a positive linear correlation between SERPINB3 and HIF-2α transcripts has been outlined, with the highest levels of HIF-2α transcripts detected in the subgroup of patients with high SERPINB3 transcripts and also characterized by early tumor recurrence and poor prognosis (Cannito et al., 2015).

Therefore hypoxia can switch on a paracrine/autocrine loop that, through the release of SERPINB3 in the extracellular environment may also affect changes in cancer cell behaviour, including EMT and increased invasiveness. This correlation between SERPINB3, hypoxia and the epithelial-mesenchymal transition may explain how SERPINB3 is commonly undetectable in the healthy liver but expressed in the early phases of carcinogenesis. Indeed, SERPINB3 has been proposed as a potential prognostic tissue marker of liver carcinogenesis (Guido et al., 2008).

The molecular mechanisms leading to HCC are still largely unknown and hepatocarcinogenesis is considerd as a multistep process; during the progression phase activation of cellular oncogenes, overexpression of growth factors, and inactivation of tumor suppressor genes may contribute to the development of the neoplastic phenotype. A meta-analysis of gene expression profiles in datasets from nine independent patient cohorts across the world allowed the identification of molecular subclasses of HCCs correlated with their histological, molecular and clinical features. In this setting, transforming growth factor β (TGF-β) signalling was identified as one of the most important features of more aggressive HCCs (Hoshida et al., 2010). Indeed, overexpression of this cytokine has been found in hepatic tumors and correlated with carcinogenesis and progression. Carcinogenesis is the late stage in the progression of chronic liver disease, and is well established that TGF-β1 is secreted by hepatic cells in response to injury, including the activated HSCs, then creating an autocrine positive feedback loop driving fibrogenesis. Along these lines, in 2008 Calabrese et al. (Calabrese et al., 2008) reported a strict correlation between TGF-β and SERPINB3 expression in parenchymal lung tissue of patients with idiopathic pulmonary fibrosis. The key pathological features of this disease resemble in some way the pathogenesis of cirrhosis, including epithelial damage, abnormal mesenchymal cell activation, and proliferation with excess of ECM deposition, driven by TGF-β1 acting as a pivotal cytokine (Calabrese et al., 2008). Two years later Turato et al. (Turato et al., 2010) described a significant correlation between TGF-β1 and SERPINB3 in

liver biopsies from a large cohort of cirrhotic patients and both proteins were found to correlate to the extent of liver fibrosis. These Authors also demonstrated that TGF-β1 expression was significantly increased in liver cell lines (HepG2 and Huh7) and primary human hepatocytes transfected to over-express SERPINB3. The parallel expression of the two molecules in idiopathic pulmonary fibrosis and in chronically damaged livers provides evidence that a common fibrogenic mechanism is likely to occur, where SERPINB3 might act as a TGF-β upstream modulator protein (Turato et al., 2010). In keeping with these data, the findings of a subsequently study support the central role of this Serpin in the upregulation of TGF-β1 (associated with cytoplasmic β-catenin accumulation) also in liver tumors, specifically in the subclass of HCCs with poorest prognosis (Turato et al., 2014). These data are of potential interest since it is well established that HCC, whatever the etiology, usually develops on the background of a cirrhotic liver and is then intimately related to the fibrogenic progression of the underlying chronic liver disease (CLD).

7. CHRONIC LIVER DISEASES

7.1 LIVER FIBROGENESIS

Chronic tissue injury leads to a sustained scarring response that gradually disrupts normal cellular functional units and eventually causes failure in multiple epithelial organs, including the liver.

Liver fibrosis is a potentially reversible wound-healing response to chronic liver injury that reflects an altered balance between liver repair and scar formation. After a liver damage a dynamic mechanism that causes release and accumulation of ECM components, attempts to limit the damage. However, when the etiologic factor persists in a scenario of chronic liver damage, the hepatic compensatory response will not be sufficient to recover completely the injured parenchyma and consequently the fibrogenic process will be constantly active, leading with the time to fibrosis, cirrhosis and eventually hepatic failure.

The primary effector cells orchestrating the deposition of extracellular matrix in normal and fibrotic liver are the hepatic stellate cells (HSC), which are activated into myofibroblast-like cells (MF) by a range of different kinds of chronic liver injury. Hepatic stellate cells (HSC), formerly defined also as Ito cells or hepatic lipocytes, are resident perisinusoidal cells in the subendothelial space between hepatocytes and sinusoidal endothelial cells. They are strategically positioned to intimately interact with hepatocytes,

endothelial cells, and nerve endings through their numerous processes extending across the space of Disse. They are responsible for the storage and metabolism of vitamin A and retinoids and for the synthesis of basal membrane like-ECM components in the sub-endothelial space of Disse. Additionally, HSCs have been suggested to play a role as liver specific pericytes, being able to respond (i.e., by either contracting or relaxing) to a number of vasoactive peptides and mediators, then affecting the caliber of liver sinusoids.

MFs

MFs represent critical cells in wound healing response since they actively regulate connective tissue remodeling by uniquely combining the ECM-synthesizing features of fibroblasts with the peculiar cytoskeletal characteristics of contractile smooth muscle cells. MFs are a heterogeneous population of α-SMA-positive cells and they can originate from fibroblasts but also from several other cell sources. Fibroblasts are known to undergo controlled and transient activation into the MF phenotype during normal tissue repair, particularly following an acute tissue injury, which is a fundamental step for restoring tissue integrity. Once restored the original condition, this transient activation is followed by the disappearance of MFs either by apoptosis or by a reversion of the phenotype. However, as it can happen under conditions of severe or chronic injury as well as of dysregulated wound healing response and in the presence of the "profibrogenic environment", the activity of MFs becomes excessive and persistent through time, significantly contributing to tissue/organ fibrosis and dysfunction (Cannito et al., 2017).

Literature data indicate that hepatic MFs originate in CLD of any etiology mainly from HSCs through a process defined as of activation/trans-differentiation. This interpretation has been emphasized by an experimental study that showed that 82% to 96% of myofibroblasts were derived from HSCs in mice treated with carbon tetrachloride (CCl4), bile duct ligation (BDL), 3,5-diethoxycarbonyl-1,4-dihydrocollidin diet and Mdr2-knockout mice (Mederacke et al., 2013).

Hepatic MFs have been also reported to originate from portal fibroblasts, which are liver resident fibroblasts located in the portal tract mesenchyme that surrounds bile ducts. These cells have been originally suggested as a significant source of MFs during the course of biliary fibrosis but fate tracing studies (reviewed in Wells et al., 2014) have somewhat reduced the emphasis on this point. However, is still interesting the hypothesis (Kinnman et al., 2002) that portal fibroblasts and MFs may represent the earliest cell populations

activated after injury to the biliary epithelium; indeed it has been suggested that HSCs and portal fibroblasts may occupy different niches, with the HSCs niche being induced by hypoxia during liver parenchymal injury and the portal fibroblast niche by the ductular reaction following biliary injury (Lemoinne et al., 2013). According to this interpretation, MFs from HSC may mediate liver wound healing whereas portal MFs may regulate scar formation (Cannito et al., 2017).

Hepatic MFs have been also described to originate from bone marrow-derived cells (BMdC): mesenchymal stem cells or from α-SMA negative BMdC defined as fibrocytes (Cannito et al., 2017). Finally MFs have been suggested to originate from either hepatocytes or from biliary epithelial cells following a process of epithelial-to-mesenchymal transition (EMT), but this hypothesis is still controversial and the matter of intense debate (Cannito et al., 2017).

Independently from their source, in a profibrogenic environment, MFs precursor are involved in the so called process of activation characterized by different morphological and phenotypical responses: high proliferative attitude; increased synthesis of ECM components, particularly fibrillar collagens (type I and IV) as well as of factors involved in ECM remodeling; ability to migrate following a chemotactic mediator; increased synthesis of growth factors and pro-inflammatory cytokines; contractility in response to vasoactive compounds; resistance to apoptosis induction.

The high proliferative attitude is the result of increased availability in the profibrogenic environment of growth factors released by surrounding cells and able to stimulate mitogenesis, as well as an increased expression of related receptors expressed by MFs. The most potent mitogen is represented by platelet-derived growth factor (PDGF), with increased expression of the corresponding α- or β-receptor subunit (PDGF-Rα or PDGF-Rβ) representing a common marker of activated precursors or MFs (Parola et al., 2008; Pinzani & Marra, 2001). Moreover, it has been demonstrated that this expression of PDGF and up-regulation of related receptors is sustained by TGF-β1. Other growth factors and mediators have been reported to stimulate proliferation of MFs including, for example, the bFGF (Fibroblast Growth Factor), AT-II (Angiotensin II), VEGF-A (Vascular Endothelial Growth Factor A) and thrombin (Parola et al., 2008; Pinzani & Marra, 2001). Several of the signals that can induce MFs to proliferate are also believed to concur to the increased survival attitude of MFs. Human HSC/MFs, for example, have been reported to be resistant to most pro-apoptotic stimuli due to Bcl-2 overexpression and up-regulation of PI3K/C-Akt signaling and to survive to high levels of ROS (Cannito et al., 2017).

The ability to migrate in the scenario of chronic tissue injury is another typical feature of activated precursor cells and/or of MFs. PDGF can be considered as the most potent chemotactic signal (Pinzani & Marra, 2001). Other chemoattractants can stimulate MFs oriented recruitment/migration, including the CC chemokine CCL2, formerly defined as monocyte chemoattractant protein 1 or MCP-1, as well as other CC-chemokines, proangiogenic peptides like VEGF-A, Angiopoietin I and Angiotensin II, CXC-chemokine receptor families (i.e. CXCR3) and ROS (Cannito et al., 2017). Moreover, all these chemotactic polypeptides activate a signaling pathway involving activation of ERK 1/2 and c-Jun-NH2 kinases 1/2 (JNK 1/2) with a redox-dependent mechanism. However, the same migratory signal pathways may be activated as a result of a significant increase in intracellular levels of ROS, even in the absence of stimulation by polypeptides (Novo et al., 2011).

Following chronic injury, myofibroblasts migrate and accumulate at the sites of tissue repair secreting large amounts of ECM and regulating ECM degradation. In this scenario TGF-β1 represents the most potent cytokine able to enhance production of fibrillary collagens (type I and III), α-SMA, laminin and fibronectin. This can be associated to a dysregulation of the expression of genes coding for enzyme involved in ECM remodeling, leading to an up-regulation of the expression of tissue inhibitors of metalloproteases (TIMPs) and a down-regulation or inefficient removal of excess fibrillary collagen by metalloproteases (MMPs). Between the mediators involved in ECM remodeling, ROS, play again an important role. MFs, epithelial cells, macrophages, endothelial cells, infiltrating leukocytes, can express multiple NOX isoforms being able to contribute to ROS generation. As an example, ROS- related mediators like the 4-hydroxy-2,3-nonenal (HNE) have been reported to up-regulate expression of pro-collagen type I and TIMP-1 and MCP-1 (CCL2) by activated HSCs, possibly through activation of specific signal transduction pathways and transcription factors, including activation of JNKs, AP-1 and NF-kB (reviewed in: Cannito et al., 2017).

A property of activated hepatic stellate cells and of myofibroblasts is their proposed role as cells contributing to innate immunity. Hepatic stellate cells have been reported to interact with different immune cell populations, with at least some of these interactions potentially promoting liver fibrogenesis. Hepatic stellate cells mediate a range of immunoregulatory effects by producing pro-inflammatory cytokines and chemokines (such as the CC-chemokine receptor 2 or CCR2, CCL2 and other chemokines like CCL3, CCL4 and CCL5); by expressing other chemokine receptors (including CCR5, CCR7, CXCR3 and CXCR7); by responding to DAMPs released by damaged epithelial cells or PAMPs

through NLRP3 inflammasome activation. Chemokine neutralization or chemokine receptor deletion in mouse liver fibrosis models has anti-inflammatory and anti-fibrotic effects by disrupting chemokine signalling in hepatic stellate cell-derived myofibroblasts and Kupffer cells (reviewed in: Cannito et al., 2017; Pellicoro et al., 2014).

Finally, HSC in physiological conditions have been described to behave also as liver specific pericytes being in strict contact with sinusoidal endothelial cells; indeed HSC/MFs synthetize and release several proangiogenic peptides like VEGF-A, Angiopoietin 1 or 2, PDGF-BB, hedgehog ligands and also express related receptors representing then also a cellular target for these mediators. These proangiogenic factors are believed to enhance the fibrogenic properties of MFs and to contribute to angiogenesis by eliciting paracrine signals affecting neighboring endothelial cells (reviewed in: Cannito et al., 2017).

Mechanisms underlying fibrosis progression

Chronic activation of the wound healing reaction is believed to represent the most common and relevant mechanism in hepatic fibrogenesis. This process is sustained by several growth factors and cytokines, many of these have already been mentioned: platelet-derived growth factor (PDGF), transforming growth factor (TGF)ß, connective tissue growth factor (CTGF), endothelin-1 (ET-1), monocyte chemotactic protein (MCP)-1, and tumour necrosis factor (TNF), to name just a few (Parola et al., 2008). More recently, the list of polypeptide mediators has been implemented by the addition of other soluble factors, including adipokines and pro-angiogenic cytokines. Where adipokines are concerned, they have been proposed as pro-fibrogenic mediators in conditions of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH). Adipokines are cytokines exclusively or predominantly secreted by the adipocytes and the best known representative adipokine, leptin, has been found to be increased in obese patients with NASH as well as in other patients with a CLD of different aetiology. Studies linking leptin to fibrogenesis range from animal to ‘in vitro’ studies showing that leptin can indeed exert a number of biological actions on HSC, including up-regulation of collagen, stimulation of cell proliferation, prevention of apoptosis and up-regulation of pro-inflammatory and pro-angiogenic cytokines (Marra & Bertolani, 2009).

Involvement of oxidative stress, reactive oxygen species (ROS) and other reactive intermediates play a predominant role in sustaining hepatic fibrogenesis and the excessive tissue remodeling. Indeed oxidative stress, presumably by promoting mitochondrial

permeability transition as well as by affecting the integrity of biological membranes, cytoskeleton as well as transcription and protein synthesis, is able to promote hepatocyte death.

Oxidative stress in CLDs results from either increased generation of ROS or other reactive intermediates, as well as by decreased efficiency of antioxidant defenses, a typical feature of CLDs. ROS and other reactive mediators such as the aldehydic end-product of lipid peroxidation 4-hydroxy-2,3-nonenal (HNE) can be released either by activated inflammatory cells or from hepatocytes directly or indirectly damaged. Generation of ROS within hepatocytes may represent a consequence of an altered metabolic state (like in NAFLD and NASH) or of ethanol metabolism (ASH), with ROS being then mainly generated by mitochondrial electron transport chain or through the involvement of selected cytochrome P450 isoforms like CYP2E1 (Novo & Parola, 2008).

ROS, HNE and other oxidative stress – related mediators released by damaged hepatocytes or activated inflammatory cells can directly affect the behaviour of human HSC/MFs and likely of other MF-like cells. ROS and HNE have been reported to up-regulate expression of critical profibrogenic genes, including pro-collagen type I, TIMP-1 and the pro-inflammatory chemokine MCP-1, possibly through activation of a number of critical signal transduction pathways and transcription factors, including activation of JNKs, AP-1 and, only for ROS, NF-kB (Novo et al., 2006; Novo & Parola, 2008; Zamara et al., 2004). A redox sensitive role should also be recognized to intracellular and NADPH-oxidase-dependent ROS generation within human and rat HSC/MFs that has been reported to occur in response to several known profibrogenic mediators, including PDGF-BB, angiotensin-II, and the adipokine leptin. ROS generated within HSC/MFs can act as positive modulators or pro-fibrogenic signalling pathways and, indeed, selective inhibition of NADPH oxidase can effectively reduce phenotypic responses of HSC/MFs (De Minicis & Brenner, 2007).

Oxidative stress may contribute to CLDs progression also by affecting immune response. Experimental studies (alcohol fed rodents) and clinical data (patients affected by ALD, chronic HCV infection or NAFLD) indicate that oxidative stress is associated with the development of circulating IgG antibodies directed against epitopes derived from protein modified by lipid peroxidation products. Titer of these antibodies correlate with disease severity and, as recently proposed for NAFLD patients, may serve as a prognostic predictor of progression of NAFLD to advanced fibrosis (Albano, 2008).

Concerning NAFLD, the long-term injury from hepatocyte triglyceride storage is responsible for the development of oxidative stress and, thus, significant intracellular generation of related reactive intermediates, with oxidative stress, may potentially favoring

NAFLD progression. Along these lines, impairment of mitochondrial β-oxidation can lead to accumulation of FFAs in hepatocytes and FFAs are substrates (ω-oxidation) and inducers of cytochrome P450 isoforms CYP2E1 and CYP4A. Both isoforms can generate ROS that, in turn, can elicit lipid peroxidation, a common hallmark of NASH. In addition, ROS may also be generated as a consequence of increased FFA oxidation in peroxisomes (β-oxidation) by Acyl-CoA oxidase (Novo & Parola, 2008). Oxidative stress may contribute to the genesis of steatosis also by negatively affecting secretion of lipoproteins (either by enhancing degradation of Apo-B100 or by affecting lipoprotein glycosylation in the Golgi apparatus) or even by interfering with the regulation of lipid synthesis by the sterol regulatory element binding protein 1 (SREBP-1) or the peroxisome proliferator-activated receptor-α (PPAR-α). Moreover, ROS have been proposed to contribute to insulin resistance (IR) itself: activation of stress-activated protein kinases by ROS can lead to impairment of the correct transduction of insulin-mediated signals through the induction of serine and threonine phosphorylation of IRS-1 (insulin receptor substrate-1) and the concomitant down-regulation of IRS-1 tyrosine phosphorylation. This has been confirmed by studies performed in hepatocytes overexpressing CYP2E1. Another potential mediators in the activation of the oxidative stress-response in NAFLD progression is the Nrf-1 gene. When using a mouse model of selective hepatic deletion of the Nrf-1 gene, the liver of these animals developed progressively all the characteristic features found in the progression of human NAFLD, including steatosis, apoptosis, necrosis, hepatitis, fibrosis and even liver cancer (Novo & Parola, 2008).

A positive feedback loop exists in which inflammatory and fibrogenic cells stimulate each other in amplifying fibrosis. Parenchymal cell damage during fibrogenesis sustains the chronic activation of inflammatory response which involves macrophages obtained from resident Kupffer cells as well as from monocytes recruited from peripheral blood and of bone marrow origin and other cells of the innate immunity like natural killer T (NKT) (Novo et al., 2015). Activated macrophages sustain liver fibrogenesis by synthetizing and releasing several pro-fibrogenic and pro-inflammatory mediators (i.e. ROS, TGFβ1, PDGF, bFGF, IL-1, TNFα MCP-1 or CCL2) (Marra & Tacke, 2014). Liver fibrogenesis is also stimulated when activated NKT cells produce mediators like IL-4, IL-13, osteopontin or Hh ligands, with an overall meaning of potentiating activation and proliferation of HSCs. But NKT cells have also an anti-fibrotic activity by inhibiting and/or killing activated HSCs when this cells mainly produce IFNγ, perforin or FasL (Novo et al., 2014).

7.2 NON-ALCOHOLIC FATTY LIVER DISEASE (NAFLD)

The obesity epidemic has grown rapidly over recent decades, with data indicating an age-adjusted obesity prevalence of over 37% in the adult population of the United States (Flegal et al., 2016). A rise in the incidence of NAFLD, characterized by the accumulation of hepatic triacylglycerol, has accompanied this increase in obesity, firmly establishing NAFLD as one of the most prevalent chronic liver diseases in Western society. The global prevalence of NAFLD is currently estimated to be 24%; it is highly prevalent in all continents, but the highest rates are reported from South America (31%) and the Middle East (32%), followed by Asia (27%), USA (24%) and Europe (23%), whereas NAFLD is less common in Africa (14%) (Younossi et al., 2017).

Several studies about NAFLD prevalence reported that both females and males are equally affected. However, some studies reveal that NAFLD is more common in men than women (Arun et al., 2006). Although studies reported that NAFLD affects people independently of the ethnicity, in the USA the prevalence of NAFLD is reported to be highest in Hispanic Americans, followed by Americans of European descent and then African Americans. The exact explanation for these ethnic differences remains unknown. Some of these differences can be explained by the genetic factors, whereas others can be explained by environmental factors such as diet, exercise and alcohol consumption (Younossi et al., 2017). The most alarming data refers to pediatric NAFLD: the prevalence of NALFD in children is 3-10%, which increases dramatically up to 40-70% among obese children. The obesity rate in children has risen from 5.0% in 1960 to 16.9% in 2009–2010. The obesity- related risk of future liver disease is alarming, as weight gain during school-years carries a higher risk of NAFLD than weight- gain in late adulthood (Younossi et al., 2017).

In USA approximately 20% of patients with NALFD, develop NASH, which is associated with a massive steatosis and inflammation. The corresponding prevalence of comorbid conditions associated with NASH in these individuals has been reported to be: obesity in 82%; type 2 diabetes mellitus in 48%; hyperlipidaemia in 82%; the metabolic syndrome in 76%; and hypertension in 70% (Younossi et al., 2017). Data regarding the prevalence of advanced forms of NAFLD and NASH in the general European population are more limited; in a meta-analysis published in 2016, the pooled NASH prevalence in Europe among patients with NAFLD with an indication for biopsy was 69.25% (Younossi et al., 2017).

Non-alcoholic fatty liver disease is diffusely characterized by the accumulation of pathological amounts of fat within the liver, which is associated, histologically, by

macrovescicular steatosis and consumption of alcohol in amount less than those that are likely to produce a liver injury (generally this is considered to be <20 g of alcohol/day for women and 30 g/ day for men) (Marchesini et al., 1999). It is now widely accepted that NAFLD represents the hepatic manifestation of the metabolic syndrome characterized by obesity (body mass index >30), hypertension, diabetes, hypertriglyceridemia and low high-density lipoprotein (HDL) cholesterol. The common thread that connects the disorders that define metabolic syndrome is the presence of underlying insulin resistance, which is considered an independent predictive factor for fibrotic and inflammatory hepatic injury. It has been widely demonstrated that NASH is strongly associated with both peripheral and hepatic insulin resistance (Marchesini et al., 2001). Obesity, type II diabetes (non-insulin-dependent) and hyperlipidemia increase drastically not only the risk and prevalence of NAFLD but also of hepatocellular carcinoma in the general population, most likely due to an advanced non-alcoholic steatohepatitis (NASH). About half of patients with hyperlipidemia were found to have non-alcoholic fatty liver disease on ultrasound examination. Hypertriglyceridemia rather than hypercholesterolemia may increase the risk of non-alcoholic fatty liver disease. A high number of patients with cryptogenic cirrhosis share many of the clinical features of patients with non-alcoholic fatty liver disease, suggesting that their cryptogenic cirrhosis may originate from an unrecognized non-alcoholic fatty liver disease (Angulo, 2002). This is due to the fact that most of patients with NAFLD have no symptoms or signs of liver disease at the time of diagnosis. Histo-pathologically, liver biopsy features from NAFLD patients include steatosis, mixed inflammatory cell infiltration, hepatocyte ballooning and necrosis, Mallory's hyaline bodies and fibrosis. A finding of fibrosis in non-alcoholic fatty liver disease suggests more advanced and severe liver injury. The combination of steatosis, infiltration by mononuclear cells and/or polymorphonuclear cells, hepatocytes ballooning and spotty necrosis is known as non-alcoholic steatohepatitis or NASH (Lackner et al., 2008).

The diagnosis of NASH is essentially based on histological examination by liver biopsy. The liver biopsy removes only about 1/50,000th of the liver which is an invasive procedure with potential life-threatening complications. The histo-pathological diagnosis of NASH has been reported to be significantly affected by inter-observer and intra-observer variabilities between pathologists. In addition, given the high number of patients affected by NAFLD, the use of liver biopsy cannot be proposed as a tool for screening general population. For these reasons, there is an urgent need to develop and validate simple, reproducible and non-invasive tests that accurately distinguish NASH from simple

steatosis and determine if possible both the stage and grade of the disease (Arora & Sharma, 2012).

Pathogenesis

In recent years it has become evident that what had initially been called the "two hit hypothesis" is probably an over-simplistic view of the NAFLD pathogenesis. The idea was that insulin resistance acted as the "first hit" by inducing lipid accumulation in the hepatocytes and increasing the vulnerability of the liver to further insults, referred to as the "second hit", that promoted hepatic injury, inflammation and fibrosis. A "multiple parallel hits hypothesis" seems more appropriate to explain the complexity of NAFLD pathogenesis, which results from numerous events originating within liver, adipose tissue, gastrointestinal tract and the muscle (Tilg & Moschen, 2010).

Lipotoxixity

Circulating free fatty acids (FFAs), which represent the major source of hepatic fat accumulation in patients with NAFLD, are mainly derived from adipose tissue lipolysis. Their plasma levels are high during fasting because they represent the fuel substrate for all tissues, and decline after feeding because of the anti-lipolytic action of insulin. The liver itself may also contribute to steatogenesis by synthesizing triglycerides from dietary carbohydrates through de novo lipogenesis but the contribution to liver fat content is less than 5% in healthy subjects and may increase to approximately 25% in NAFLD patients (Lonardo et al., 2017).

The unbalance between caloric intake and expenditure determines the expansion of fat depots, which become inflamed and insulin resistant. In the presence of adipose tissue insulin resistance, FFA levels are high, despite high levels of circulating insulin, because of the resistance to the anti-lipolytic action of this hormone. Plasma FFAs are reabsorbed in various organs where, if not oxidized, they accumulate under the form of triglycerides within intra-cytoplasmic lipid droplets, and some lipid intermediates, such as or diacyl-glycerols (DAG), promote cell lipotoxicity and mitochondrial dysfunction (Petta et al., 2016). Another lipid having a role in NAFLD is ceramide that impair insulin capability to stimulate glycogen synthesis and suppress gluconeogenesis, through protein kinase-C epsilon (PKCε) activation (Chaurasia et al., 2015). Among toxic lipids, saturated fatty acids have been shown to be elevated in NASH patients and

induce inflammation and hepatocyte apoptosis by activating JNK and mitochondrial pathways (Leamy et al., 2013). In contrast, unsaturated fatty acids do not affect cell viability and an increase in their content leads to enhanced hepatic synthesis of TG. In turn, TG accumulation is not toxic but may protect the liver from the excessive deposition of toxic TG precursors (McClain et al., 2007). Omega-3 polyunsaturated fatty acids (PUFAs) plasma levels are reduced in patients with NASH (Sanyal et al., 2015). However, pharmacologic supplementation did not induce an amelioration of the histologic picture of NASH, and in an experimental model it was even associated with more severe damage (reviewed in Caligiuri et al., 2016).

Lipotoxicity leads to mitochondrial dysfunction and endoplasmic reticulum stress. Mitochondrial β oxidation is the dominant oxidative pathway for the disposition of fatty acids under normal physiologic conditions but can also be a major source of ROS (Neuschwander-Tetri, 2010; Unger, 2002). Several lines of evidence suggest that mitochondrial function is impaired in patients with NASH. Patients with steatohepatitis have ultrastructural mitochondrial lesions, including linear crystalline inclusions in mega-mitochondria (Lotowska et al., 2014). This mitochondrial injury is absent in most patients with simple steatosis and in healthy subjects. The mitochondrial defects in patients with NAFLD may be indicative of defective oxidative-phosphorylation, as well as these patients also have reduced mitochondrial respiratory chain (MRC) activity (Pérez-Carreras et al., 2003). MRC dysfunction can directly lead to the production of ROS. If electron flow is interrupted at any point in the respiratory chain, the preceding respiratory intermediates can transfer electrons to molecular oxygen to produce superoxide anions and hydrogen peroxide. As the oxidative capacity of the mitochondria becomes impaired, cytosolic fatty acids accumulate. Alternative pathways in the peroxisomes (β oxidation) and in microsomes (ω oxidation) are activated, resulting in the formation of additional ROS (Browning & Horton, 2004). ROS can attack polyunsaturated fatty acids (PUFAs) and initiate lipid peroxidation within the cell, which results in the formation of aldehyde by-products such as trans-4-hydroxy-2-nonenal (HNE) and malondialdehyde (MDA) (Parola et al., 1998). These molecules impair nucleotide and protein synthesis, deplete the natural antioxidant glutathione, increase production of the proinflammatory cytokine TNF-α, promote influx of inflammatory cells into the liver, and activate stellate cells, leading to collagen deposition, fibrosis, and the perpetuation of the inflammatory response (Browning & Horton, 2004).

Furthermore, fatty acids can induce not only oxidative stress but also endothelial reticulum (ER)-stress, which refers to any perturbation of the ER homeostasis that affects the assembly of proteins in the ER lumen and determine accumulation of improperly folded protein. This evokes a physiological response termed Unfolded Protein Response (UPR) that consist in the activation of an intracellular signaling leading to an increased transcription of ER-resident chaperones and a decreased overall protein synthesis. Normally, the UPR contributes to restore the ER function, but when it is inadequate or insufficient, it is associated with the activation of the apoptotic pathway and cell death. Several studies have been shown that ER-stress and UPR-mediated signaling contribute to the development of steatosis and may play a central role in NAFLD/NASH (Kapoor & Sanyal, 2009; Schröder & Sutcliffe, 2010).

Inflammation

Chronic inflammation is one of the key factors distinguishing a simple steatosis from steatohepatitis and it plays an important role by contributing to stimulate fibrosis and angiogenesis during NASH progression. Steatosis leads to increased signaling of the transcription factor NF-κβ (nuclear factor-kappaβ) through the upstream activation of IKKβ. The activation of NF-κβ induces the production of pro-inflammatory mediators like TNF-α (tumor necrosis factor-alpha), IL-6 (interleukin-6) and IL-1β (interleukin-1β). These cytokines have been reported to play a role in hepatic insulin resistance and contribute to the recruitment and activation of Kupffer cells (Cobbina & Akhlaghi, 2017). Moreover, some member of the TNF-α superfamily receptor, like TNF-related apoptosis-inducing ligand (TRAIL-R) and tumor necrosis factor receptor (TNFR) are upregulated in NASH. It has been shown that TRAIL-R-deficient mice display reduced steatosis, inflammation and fibrosis in association with lower hepatocyte apoptosis (Caligiuri et al., 2016). Another signaling pathways involved in development of chronic inflammation during NAFLD is the one activated by JNK: liver-specific knockdown of JNK-1 shown a reverse effect on insulin-resistance, improves hepatic steatosis and increases the amount of circulating triglycerides (Singh et al., 2009).

Among the different type of immune cells, Kupffer Cell (KC) activation is critical in NASH and precedes the recruitment of other circulating monocyte/macrophage cells. KC are the resident macrophages of the liver and function in both innate and adaptive immunity as active phagocytosing agents and antigen-presenting cells to T-cells. KC as well as recruited monocytes/macrophages may acquire two polarization phenotypes currently defined as M1 (pro-inflammatory or classically activated) and M2 (alternatively

activated or of resolution) macrophages. Differentiation toward a pro-inflammatory 'M1 phenotype' is driven by pathogen-associated molecular patterns (PAMPs) and damage-associated molecular patterns (DAMPs) interacting with toll-like receptors (TLR) or other pattern-recognition receptors (PRRs), resulting in the increased expression of pro-inflammatory cytokines IL-1β, IL-12, TNF-α, CCL2 and CCL5. Some cytokines (i.e., CCL2 and CCL5), can induce HSC activation, initiating a fibrogenic response. Different experimental models of NASH have clarified that CD4+ and CD8+ T-, B-lymphocytes and natural killer T (NKT) cells are also recruited in the injured liver (Sutti et al., 2016). Moreover, experiments performed in murine models of NASH and in human cells isolated from patients undergoing hepatic resection have identified a subtype of dendritic cells (DCs) able to produce various cytokines such as TNF-α, IFN-γ, IL-6, IL-4, and IL-2. DCs also showed a response to TLR ligation and a strong T-cell proliferation and seemed to activate natural killer and natural killer T cells (Ibrahim et al., 2012). Accordingly, other findings have indicated that by preventing the accumulation of DCs subtypes expressing high levels of inflammatory factors one can limit liver injury in experimental NASH (Sutti et al., 2015). Despite these properties, the role of liver dendritic cells during NAFLD evolution remains an open issue.

One of the most recently identified contributor to the cross talk between hepatocytes and non-parenchymal cells during NAFLD is represented by the multiprotein platform complex nucleotide-binding oligomerization domain-like receptor protein 3 (NLRP3) inflammasome. Full activation of NLRP3-inflammasome, via NF-κB, is induced by TLRs together with signals linked to cellular damage and results in secretion of mature IL-1β and IL-18. These cytokines, acting on different cell types, produce inflammatory signals in the injured liver as well as in the adipose tissue and intestine, triggering steatosis, insulin resistance, inflammation and cell death (Caligiuri et al., 2016). Recently, our laboratory has provided evidence that extracellular vesicles released by fat-laden cells, thus undergoing lipotoxicity, can directly up-regulate NLRP3 inflammasome in both hepatocytes and macrophages, resulting in a significant increase in IL-1β release (Cannito et al., 2017b). Inflammasome responses can also cause pyroptosis, a peculiar and rapid pro-inflammatory form of cell death which is known to occur also in NASH (Kubes & Mehal, 2012).

Adipokines

Among the cytokines, the adipose tissue-secreted adipokines are linked to progression from NAFLD to NASH. Adiponectin is a cytokine that is mostly produced by adipocytes,

it is involved in the regulation of glucose and lipid metabolism, as well as in inflammation by inhibiting NF-κB and TNF-α production in macrophages. Consistent with these data, adiponectin serum levels are inversely related to insulin resistance and are lower in obese subjects and patients with established insulin resistance, in type 2 diabetes, NAFLD/NASH and hypertension. Conversely, higher serum leptin levels were associated with an increased severity of NAFLD. Leptin is another adipokine primarily secreted from adipose tissue and, like adiponectin, controls fat catabolism and glucose production activating central neural pathways and increasing hepatic AMPK (reviewed in Parola & Marra, 2011). In obese mice, leptin causes weight loss, increasing energy expenditure and fatty acid oxidation, reducing appetite and triglyceride synthesis and counteracting the lipogenic action of insulin. It has been established that this cytokine exerts a protective effect in early disease while as NAFLD proceeds, it acts as a pro-fibrogenic and inflammatory factor (reviewed in Caligiuri et al., 2016).

Literature data suggest that Leptin may exert its pro-inflammatory activity by the impairment of NO-related vassal relaxation, via increased oxidative stress, and by increased endothelin expression, by potentiating the effect of angiotensin II and by increasing the expression of adhesion molecules (e.g. VCAM-1, ICAM-1 and E-selectin) (Petta et al., 2016). Other adipokines, such as resistin (RETN), visfatin (NAMPT) and retinol binding protein 4 (RBP4) also appear as promising potential NAFLD mechanistic markers.

Microbiota

Another mechanism involved in NASH pathogenesis is represented by changes in gut microbiota composition. In NAFLD an increased Firmicutes/Bacteroidetes ratio and changes in metagenomic-based functional aspects of intestinal microbiota have all been described. A high-fat diet increases the portion of intestinal Gram- negative endotoxin-producing bacteria, resulting in higher rates of bacterial translocation, and accelerated fibrogenesis by enhancing HSC activation in CCl_4 and BDL mice (De Minicis, Rychlicki, et al., 2014). Moreover, some study showed that the transfer of gut microbiota from obese to lean individuals induced in the recipients, the same metabolic alterations of the donors. Microbiota may contribute to the development and progression of NAFLD by triggering different signaling pathways, by increasing the efficiency of caloric extraction from the food, and by inducing translocation of bacterial products *via* increased gut permeability (Lonardo et al., 2017). According to this, NAFLD patients often exhibit a disruption in the

intestinal barrier and this can expose the liver to increased levels of bacterial products such as LPS that can activate TLRs and induce pro-inflammatory pathways, which, in turn, can further contribute to liver disease development and progression.

8. HRGP

Concerning the molecular factors involved in the progression of NAFLD, recent research has outlined the role of histidine-rich glycoprotein (HRG). HRG is a member of the cystatin superfamily, produced in the liver and traceable in vertebrates plasma (100–150 mg/l in human plasma) and that can actively be internalized by different tissues (Ronca & Raggi, 2015). The protein consists of multiple domains, with a central histidine-rich region (HRR) that is flanked by two proline-rich regions (PRR1 and PRR2). As a glycoprotein, HRG has two N-glycosylation modification sites in the N-terminal domain (Asn 63 and Asn 125). Despite the function of the N-glycosylated modification of HRG is not completely understood, some reports found that glycosylation at the N-terminal might play a key role in the interaction between HRG and its target (e.g., HRG cannot inhibit Erk1/2 phosphorylation without glycosylation) (Zhang et al., 2015). HRG can interact with a variety of ligands and seems to be involved in the blood coagulation and fibrinolysis systems as well as in regulation of angiogenesis, in proliferation and interacts with components of the immune system. Due to the high content of histidines within the central histidine-rich region, HRG can interact with divalent metal ions and heme. It appears that HRG undergoes a conformational change after Zn^{2+} binding for example, providing a regulatory mechanism for the function of HRG. Other ligands include heparin, heparanase, heparan sulfate, plasminogen, and thrombospondins-1 and -2 (Jones, Hulett, & Parish, 2005). Old literature data suggested a rather complex modulation of HRG in liver injury because HRG levels were found to be elevated in mild liver cirrhosis and decreased in moderate and severe cirrhosis (Gram et al., 1985). More recently, Zhang et colleagues (2015) evaluated the role of HRG in HCC and showed that HRG was down-regulated in HCC tissues and that the overexpression in hepatic tumor cells of HRG resulted in a decrease of cell proliferation, colony formation and tumor growth, also resulting in the promotion of cancer cell apoptosis, suggesting a tumor-suppressor role for HRG in HCC (Zhang et al., 2015). These data seems in line with results from other studies that have described low levels of HRG in other types of cancers such as endometrial carcinoma and ovarian cancer. Along these lines, HRG treatment can increase apoptosis and reduce

proliferation also in fibrosarcoma and suppress endothelial cell migration; accordingly, these Authors described an increased tumor-growing ability of fibrosarcoma and pancreatic carcinomas in HRG-deficient mice as compared to normal mice (Zhang et al., 2015). The potential relevance of HRGP in supporting the evolution of NAFLD steams from a recent report by Bartneck et al. (2016) who reported that $HRGP^{-/-}$ mice showed a significant reduction in the recruitment of hepatic macrophages, and particularly of those M1 polarized, while the remaining macrophages displayed markers of M2 polarization. This was confirmed by in vitro experiments showing that purified human HRGP is able to induce M1 macrophage differentiation. Moreover, $HRGP^{-/-}$ mice were significantly protected from liver injury and fibrosis in mice with MCD-induced NASH. Moreover, in liver tissues from NASH as well as HCV-related patients, HRG resulted up-regulated in hepatocytes and associated with M1 polarization of adjacent macrophages (Bartneck et al., 2016), overall suggesting that HRG released by hepatocytes has the potential to sustain inflammatory response (i.e., in particular M1 macrophages) and then fibrogenic progression of chronic liver diseases.

AIM OF THE STUDY

The obesity epidemic has grown rapidly over recent decades and a rise in the incidence of NAFLD has accompanied this increase in obesity, firmly establishing NAFLD as one of the most prevalent chronic liver diseases in Western countries (Flegal et al., 2016). Progressive NAFLD includes a spectrum of relevant metabolic features like insulin resistance and histopathological changes including steatosis/steatohepatitis, fibrosis, cirrhosis and hepatocellular carcinoma (HCC). It has been established that hypoxia can play a role in the pathogenesis and in the progression of this diseases mainly through the involvement of hypoxia-inducible transcription factors (HIFs) and activation of related adaptive transcriptional responses (Nath & Szabo, 2012). Concerning HIFα subunits HIF-1α is ubiquitously expressed in almost all tissues and organs, whereas HIF-2α expression is limited to some specific tissues such as the endothelial cells, hepatocytes, kidney tubular cells and enterocytes (Talks et al., 2000). In the liver, HIF-2α has been reported to be able to modulate lipid accumulation and insulin sensitivity by modulating hepatic fatty acid oxidation as well as insulin and glucagon signaling (Ramakrishnan et al., 2016; Rankin et al., 2009; Taniguchi et al., 2013; Wei et al., 2013). Along these lines, an interesting experimental mechanistic study has been performed some years ago in a mouse model characterized by hepatocyte-specific deletion of Von Hippel-Lindau (VHL) expression, a condition leading to overexpression of HIFs and resulting by itself (i.e., in the absence of any dietary regimen) in a very significant fatty liver (Qu et al., 2011). In particular, when hepatocyte-specific deletion of VHL was combined with either hepatocyte-specific deletion of HIF-1α and/or HIF-2α, results obtained in these double transgenic mice suggested that HIF-2α had a major role in the development of spontaneous steatosis. Moreover, in the presence of a short protocol (2 weeks) of ethanol administration, HIF-2α was hypothsized to be possibly also related to liver inflammation and fibrosis. Indeed, the ethanol-related increase in steatosis and in some markers of inflammation and fibrosis was completely prevented in hepatocyte-specific *Vhl-Hif-2α* double knockout mice, but not in hepatocyte-specific *Vhl-Hif-1α* double knockout mice (Qu et al., 2011). Although these data were suggesting a possible critical role for HIF-2α activation in a scenario of potential interest in relation to human NAFLD, they were not performed in association with a dietary model for experimental NAFLD induction. Indeed, these published experiments then did not offer mechanistic information on the role of hypoxia and HIFs in relation to NAFLD progression. This is relevant since mechanisms underlying NAFLD progression are still largely unclear in a disease for which, despite its high incidence in the

general population, no reliable biomarkers are available to predict the individual risk of progression and/or HCC development as well as validated therapeutic strategies. Based on these consideration, the present study has been designed in order to specifically investigate the biological function of hypoxia, HIF-2α and HIF-2α-related potential target genes and mediators in the fibrogenic progression of NAFLD and possibly of other chronic liver diseases of different etiology.

In particular, the experiments described in the first published study were designed to mechanistically characterize in vivo the role of HIF-2α in the development and progression of NAFLD. The role of HIF-2α in this specific study has been evaluated in the development of hepatic fibrosis by employing a mouse model with hepatocyte-specific deletion of HIF-2α (hHIF-2α $^{-/-}$). These mice were subjected to well established experimental models of progressive and dietary-induced progressive NAFLD/NASH: the methionine and choline-deficient (MCD) diet and the choline-deficient L-amino acid-defined (CDAA) diet. This study outlined a major pathogenic role in NAFLD progression for hepatocyte- and HIF-2α-dependent release of the hepatokine histidine-rich glycoprotein (HRG), a hepatokine recently reported to support inflammation during either experimental chronic liver injury as well as corresponding human diseases (Bartneck et al., 2016).

Data obtained in the past from our group and collaborating laboratories have also identified the serine protease inhibitor SerpinB3 (SB3) as a peptide mediator released by hepatocytes during HCC in a HIF-2α-dependent manner (Cannito et al., 2015). Moreover, SB3 expression was found to be positively correlated with that of TGF-β in chronically damaged livers as well as to the extent of liver fibrosis (Cristian Turato et al., 2010), with additional in vitro experiments suggesting that SB3 might act as a TGF-β upstream pro-fibrogenic mediator (Cristian Turato et al., 2010). Based on these evidences, a second aim of this thesis, accomplished in paper n. 2, was to investigate the possible pro-fibrogenic role of SerpinB3 in the progression of experimental models of CLD as well as in liver specimens from NAFLD and chronic HCV human patients.

EXPERIMENTAL MOUSE MODELS OF HEPATIC FIBROSIS

Murine models constitute the bulk of research in preclinical liver disease pathology, with some of these models exhibiting a satisfying degree of translatability to clinical conditions. In the studies included in the present thesis we have used three different in vivo experimental murine models of CLD leading to chronic injury, chronic inflammation and liver fibrosis: i) the methionine- and choline-deficient (MCD) diet and ii) the choline-deficient L-aminoacid-defined (CDAA) diet, as established models of experimental progressive NAFLD, as well as iii) the model of chronic carbon tetrachloride (CCl_4) administration. All animal protocols were approved by the local University Commissions for Animal Care (Università del Piemonte Orientale, Novara and University of Padova) and by Italian Ministry of Health and the experiments complied with national ethical guidelines for animal experimentation. Hereafter, the three different experimental murine models employed in the present thesis are described and discussed. More details can be found in the publications generated in these years.

1. The methionine- and choline-deficient (MCD) diet.

From the histopathological point of view, this experimental model is known to reproduce several histopathological changes detected in human NAFLD/NASH patients. This diet contains a high amount of fat and sucrose (10% fat kcal and 40% carbohydrate kcal) and it is deficient in methionine (essential amino acid and important methyl donor) and choline (precursor for de novo phosphatidylcholine synthesis and hepatocyte export of triglycerides via VLDL packaging) (Vance et al., 1985). An isocaloric diet, methionine and choline supplemented (MCS), is usually administered to controls animals.

As mentioned, the main advantage of this dietary regimen is to result in the induction of histological and pathogenic features of progressive NAFLD resembling those observed in humans, including steatohepatitis and fibrosis, oxidative stress, altered levels of cytokines and adipokines, with most critical changes developing quite rapidly. Mice fed on MCD diet develop hepatic macrovescicular steatosis and infiltration of inflammatory cells after 1–3 weeks of feeding with robust perisinusoidal fibrosis becoming detectable between from 4 to 8 weeks of feeding (Sahai et al., 2004). A major bias of the MCD diet model consists in the induction of hypophagia and hypercatabolism resulting in significant body-weight loss (20–40%) accompanied by a proportional loss of liver mass. Moreover, in this model we have a lack of obesogenic/metabolic effects and, accordingly, the lack of the

raise of the insulin-resistant phenotype that characterizes the progression of human NAFLD/NASH (Hansen et al., 2017).

In our experiments the MCD diet was given up to 8 weeks to the following experimental groups of mice (all on the C57/BL6 genetic background): i) mice carrying or not (i.e., control littermates) a hepatocyte-specific deletion of HIF-2α, thereafter defined as hHIF-2α $^{-/-}$ mice, obtained by breeding HIF-2α fl/fl mice with mice expressing the Cre-recombinase under the control of the Albumin promoter (Alb/Cre mice) (Jackson Laboratories, Bar Harbor, Maine, USA). Groups of control animals (carrying or not hepatocyte-specific deletion of HIF-2α) were fed with the corresponding methionine/choline sufficient (MCS) diet; ii) transgenic mice overexpressing in murine hepatocytes human SERPINB3 (TG/SB3 mice) and their related wild type (WT) mice, a mouse model previously characterized (Villano G. et al, 2009). The latter murine experiments using TG/SB3 and WT mice were performed in the collaborating laboratory of Prof. Pontisso, at the Dept. Medicine, University of Padova, Italy. The sequence of human SB3 (-7/+1238) was inserted into the pcDNA3 plasmid vector under the α1 -antitripsin (α1AT) promoter.

2. The choline-deficient and L-aminoacid defined (CDAA) diet.

The catabolic profile induced by MCD diet limits the clinical translatability of results and for this reason we have performed the same experiments using a second and different model of progressive NAFLD. The CDAA diet, originally used in rat models, is based on the administration of a choline-deficient and L-aminoacid-defined diet or, in control mice, choline-supplemented and L-aminoacid-defined diet (CSAA). Both the CDAA and CSAA diets consist of L-aminoacids (except glycine that is not optically active) and are essentially isocaloric (4.32 and 4.27 kcal/g for CDAA and CSAA, respectively) with equal content in total aminoacids (proteins). The CDAA diet contains 6.5 mg/kg of choline and 1.75 g/kg of methionine. The CSAA is supplemented with 14.48 g/kg of choline (Sakaida et al., 1998).

The induction of progressive NAFLD/NASH in mice fed on the CDAA diet resulted in a slower appearance of main histopathological changes as compared to the MCD diet protocol, with experimental time points for collection of samples at 12 and 24 weeks of CDAA diet feeding. Mice fed on CDAA diet do not lose but rather increase their body weight through the time and start to develop macrovescicular steatosis from week 3 and at 12 weeks the liver is already presenting steatohepatitis and the onset of early fibrosis, with

the latter changes being more significant at 24 weeks (De Minicis et al., 2014; Hansen et al., 2017).

3. Comparative analyses performed on the MCD and CDAA murine models of NAFLD.

In a first set of in vivo preliminary experiments we decided to run in parallel the two dietary models of progressive NAFLD and we fed C57/BL6 wild type (WT) mice on either MCD diet (and MCS control diet) for 4 or 8 weeks or CDAA diet (and CSAA control diet) for 12 and 24 weeks to then compare major data and histopathological changes obtained in the two different models. These studies (MCD and CDAA models) were carried out in accordance with the guidelines for the care and use of laboratory animals and mice were bred at the Animal Care Facility of the University of Eastern Piedmont (Università del Piemonte Orientale, Dept. Health Sciences, Novara).

Although absolute (i.e., quantitative) levels of hepatic steatosis seem to overlap in the two dietary models at the defined experimental time points, as evaluated by means of liver TG content (at 4-8 wks in MCD protocol and 12-24 wks in CDAA protocol) (Figure 1A), it should be underlined that the CDAA diet protocol caused a lower degree of hepatocellular injury as compared to the MCD diet one (Figure 1B). However, the lower extent of hepatic injury in the CDAA diet model was favoring a more regular progression of chronic liver disease. According to decreased ALT levels, the CDAA diet resulted in an inflammatory reaction that appears to be more contained (as for transcript levels of some pro-inflammatory mediators) but more progressive over the time. For example, levels of CCL2 and CXCL10 transcripts (Figure 2) progressively increased between 12 and 24 weeks with the CDAA diet, while with the MCD diet they reached higher values as soon as after 4 weeks to then decline at 8 weeks. The analysis of collagen deposition (Sirius Red staining, Figure 3) and of transcripts levels of pro-fibrogenic genes showed that liver fibrosis at 24 weeks in the CDAA murine model was compatible with the scenario observed in mice fed on MCD diet for 8 weeks; however, α-SMA liver transcripts levels (Figure 4) and the number of hepatic MFs (Figure 5) were higher in the liver of mice fed on CDAA diet as compared to liver samples from mice fed on the MCD diet. The data obtained in the present thesis essentially confirm those previously reported for mice fed on CDAA diet (De Minicis, Agostinelli, et al., 2014), overall also confirming that the CDAA protocol is more compatible with critical features detected in human NAFLD/NASH patients.

Figure 1

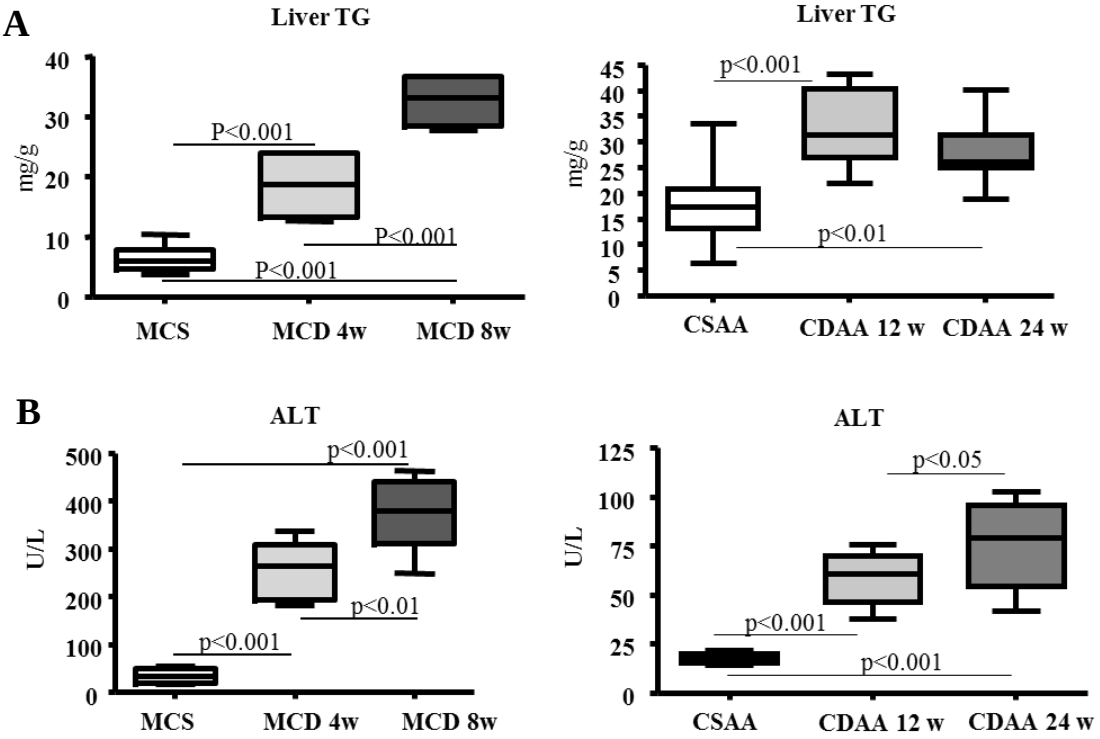

Figure 1. Mice fed with MCD diet up to 8 weeks and with CDAA diet up to 24 weeks were evaluated for the following parameters: **(A) liver steatosis, as evaluated** by measuring hepatic TG content, **(B)** serum levels of alanine (ALT)**. Control mice were fed with MCS diet and CSAA diet.** The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 2

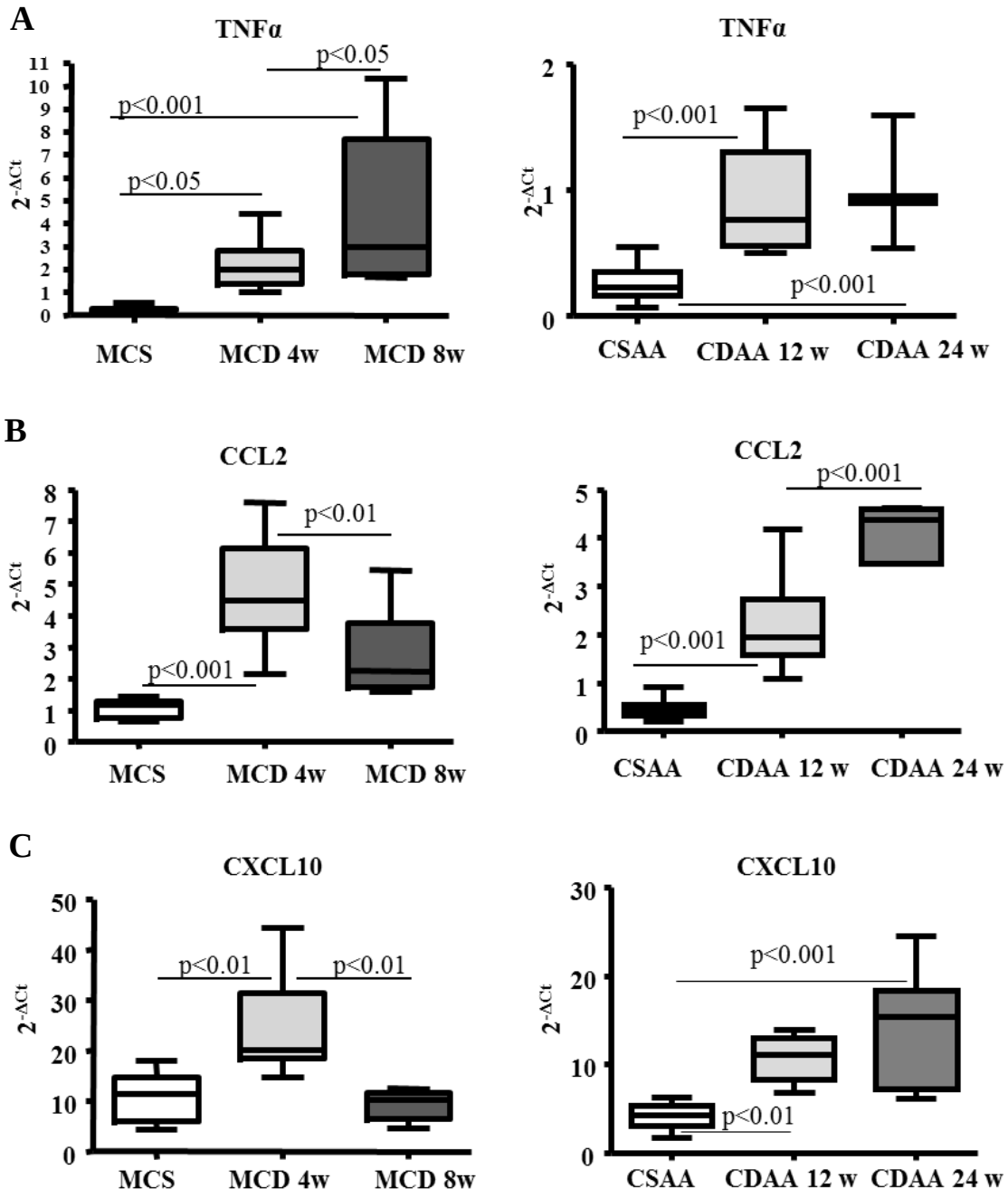

Figure 2. Liver expression of inflammatory markers TNF-α (**A**), CCL2 (**B**) and CXCL10 (**C**), as evaluated by quantitative real-time PCR (Q-PCR) in mice treated with control diet or MCD for 4-8 weeks (left panel); and mice treated with control diet (CSAA) or CDAA for 12-24 weeks (right panel). The mRNA values were normalization to the β-actin gene expression and are means ± SD of 6-8 animals per group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 3

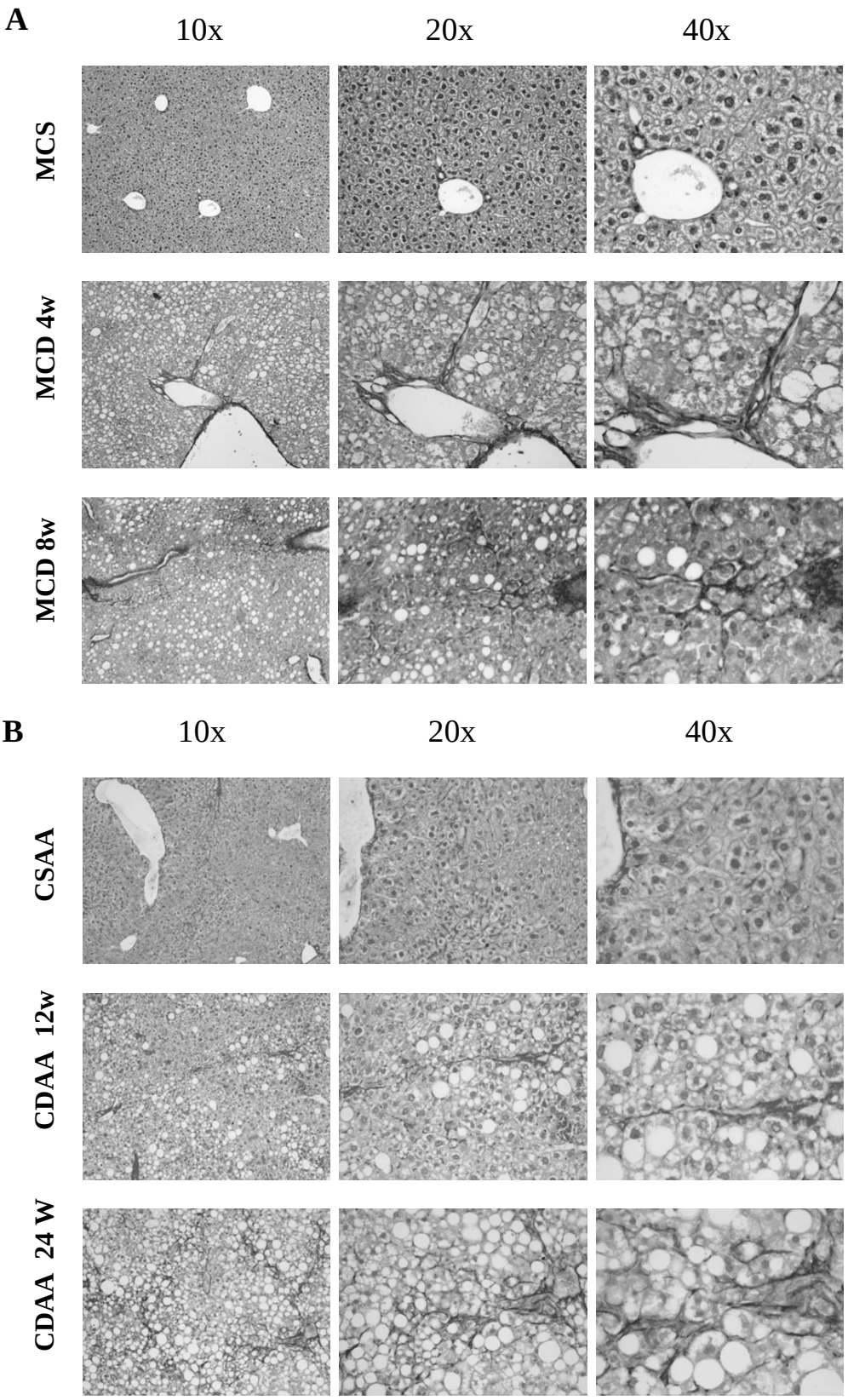

Figure 3. Liver fibrosis was evaluated morphologically in MCD (**A**) and CDAA mice (**B**) and mice fed with control diets (MCS and CSAA, respectively) by Sirius red staining. Original magnification as indicated.

Figure 4

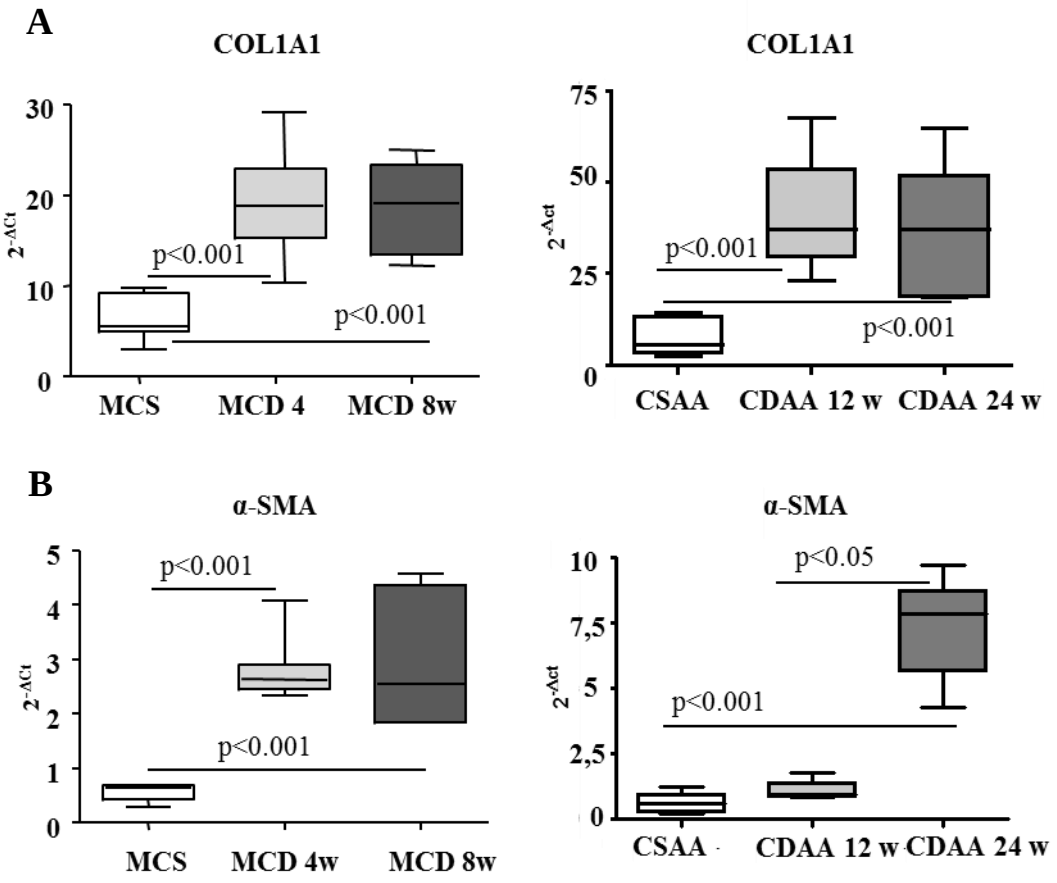

Figure 4. Liver expression of transcript levels of pro-fibrogenic genes collagen 1A1 (**A**) and α-sma (**B**), as evaluated by quantitative real-time PCR (Q-PCR) in mice treated with control diet or MCD for 4-8 weeks (left panel); and mice treated with control diet (CSAA) or CDAA for 12-24 weeks (right panel). The mRNA values were normalized to the β-actin gene expression and are means ± SD of 6-8 animals per group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figura 5

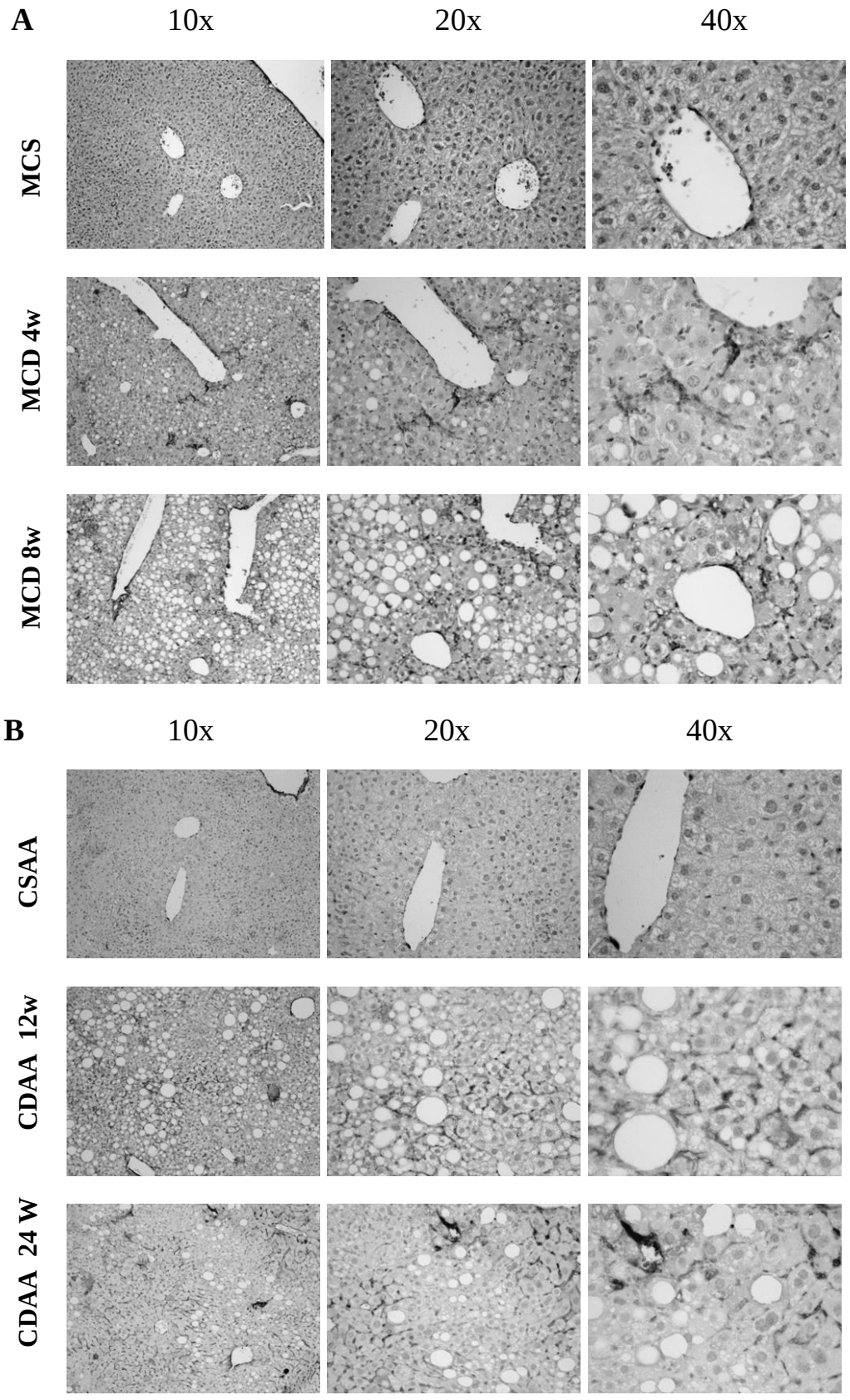

Figure 3. Liver fibrosis was evaluated morphologically in MCD (**A**) and CDAA mice (**B**) and mice fed with control diets (MCS and CSAA, respectively) by immunohistochemistry for α-SMA. Original magnification as indicated.

4. Carbon tetrachloride (CCl_4) chronic administration.

Chronic parenchymal liver injury induced by repeated administration of carbon tetrachloride (CCl_4) has been extensively used in the last decades as a model to investigate mechanisms of hepatic fibrosis progression. This is an interesting model since chronic administration of the hepatotoxin can also progress to liver cirrhosis and the development of HCC. CCl_4 is a typical liver-targeted toxins which is primarily taken up by hepatocytes and then metabolized to reactive intermediates that in turn can cause oxidative stress – dependent necro-inflammation and excess activation and proliferation of pro-fibrogenic HSCs. The first appearance of histological fibrosis and scarring fibers is usually observed after 2 to 3 weeks of CCl_4 treatment whereas true bridging fibrosis can be observed after 4 to 6 weeks of continuous treatment (Liedtke et al., 2013).

In the present thesis, transgenic mice (TG/SB3) for human SERPINB3 (described above) and related wild type (WT) mice (all on the C57/BL6 background) were employed; these mice (6–8 weeks of age) were submitted to the protocol of chronic liver injury (as described in Wang et al., 2007) requiring chronic administration of the hepatotoxin for 10 weeks trough intraperitoneal injections of 0.5 μL/g per gram body weight of a CCl4/olive oil mixture (20% solution) twice a week. Mice were killed always at 72 hours following the last injection. Controls WT and TG mice received injections of olive oil. These in vivo experiments were performed at the Dept. of Medicine, University of Padova, by the collaborating group of Prof. Pontisso who provided us liver specimens and other biological samples. The study was carried out in accordance with the guidelines for the care and use of laboratory animals and mice were bred at the Animal Care Facility of the Experimental Surgery Division of the University of Padova.

PATIENT POPULATIONS

In the first study we employed a cohort of 27 patients with biopsy proven NAFLD prospectively enrolled in the Division of Gastroenterology of the Torino University Hospital. The liver biopsy was performed to confirm the diagnosis of NAFLD in patients after exclusion of liver disease of other etiology, such as alcohol-induced or drug-induced liver disease, autoimmune or viral hepatitis, and cholestatic or genetic liver disease. Clinical and anthropometric data were collected at the time of liver biopsy and tissues were evaluated for the severity of steatohepatitis and fibrosis according to Kleiner et al. (Kleiner et al., 2005). Liver biopsies from NAFLD patients ranging from early disease (staged F0-F1) to more advanced conditions of fibrosis (staged F2-F3) or cirrhosis (F4). The clinical and biochemical features of the patients are reported in the supplementary material of paper n.1 (supplementary table 1). In the second study we employed paraffin liver sections of cirrhotic specimens derived from patients with HCV-related liver cirrhosis (METAVIR F4) and referring to the Division of Gastro-Hepatology of the University of Turin. Control human liver tissue (as defined for normal histological structures observed in hematoxylin and eosin (H&E) sections, and absence of inflammation in the portal tract and parenchyma, was obtained from resection samples taken at a distance of more than 5 cm from the border of liver metastasis of colon carcinoma (n=5). The studies were approved by the ethics committees of the University Hospitals of Torino in accordance with the Helsinki Declaration and an informed consent has been obtained by all patients.

RESULTS

1. PAPER n.1

"Hypoxia-inducible factor 2α drives nonalcoholic fatty liver progression by triggering hepatocyte release of histidine rich glycoprotein."

Elisabetta Morello, Salvatore Sutti, Beatrice Foglia, Erica Novo, Stefania Cannito, Claudia Bocca, Martina Rajsky, Stefania Bruzzì, Maria Lorena Abate, Chiara Rosso, Cristina Bozzola, Ezio David, Elisabetta Bugianesi, Emanuele Albano, Maurizio Parola.

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Hypoxia-inducible factor 2α drives nonalcoholic fatty liver progression by triggering hepatocyte release of histidine rich glycoprotein

Elisabetta Morello[1]*, **Salvatore Sutti**[2,3]*, Beatrice Foglia[1], Erica Novo[1], Stefania Cannito[1], Claudia Bocca[1], Martina Rajsky[1], Stefania Bruzzì[3], Maria Lorena Abate[2], Chiara Rosso[2], Cristina Bozzola[3], Ezio David[4], Elisabetta Bugianesi[2], Emanuele Albano[3], Maurizio Parola[1§]

[1]Dept. Clinical and Biological Sciences, University of Torino, Italy.

[2]Dept. Medical Sciences, University of Torino, Italy.

[3]Dept. Health Sciences and Interdisciplinary Research Center for Autoimmune Diseases, University Amedeo Avogadro of East Piedmont, Novara, Italy.

[4]Pathology Unit, S. Giovanni Battista Hospital, Torino.

*These Authors contributed equally to this study

[§]Corresponding Author /Contact Information: Maurizio Parola – maurizio.parola@unito.it

Author names

Elisabetta Morello elisabetta.morello@unito.it

Salvatore Sutti salvatore.sutti@med.uniupo.it

Beatrice Foglia beatrice.foglia@unito.it

Stefania Cannito stefania.canito@unito.it

Erica Novo erica.novo@unito.it

Claudia Bocca claudia.bocca@unito.it

Martina Rajsky rajsky.martina@gmail.com

Stefania Bruzzì stefania.bruzzi@med.uniupo.it

Maria Lorena Abate marialorena.abate@unito.it

Chiara Rosso chiara.rosso@unito.it

Cristina Bozzola cristina.bozzola@med.uniupo.it

Ezio David ezio.david@unito.it

Elisabetta Bugianesi elisabetta.bugianesi@unito.it

Emanuele Albano emanuele.albano@med.uniupo.it

Maurizio Parola maurizio.parola@unito.it

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FOOTNOTE PAGE

Contact Information: Prof. Maurizio Parola, Dept. Clinical and Biological Sciences, Unit of Experimental Medicine and Clinical Pathology, University of Torino

Corso Raffaello 30, 10125 Torino, Italy.

Phone +39-011-6707772 Fax +39-011-6707753

e-mail maurizio.parola@unito.it

List of abbreviations. NAFLD, nonalcoholic fatty liver disease; CLD, chronic liver disease; NASH, nonalcoholic steatohepatitis; HCC, hepatocellular carcinoma; HIFs, Hypoxia-inducible factors; HIF-1α, hypoxia-inducible factor-1α; HIF-2α, hypoxia-inducible factor-2α; HIF-1β, hypoxia-inducible factor-1β; ROS, reactive oxygen species; HRGP, histidine-rich glycoprotein; MCD diet, methionine/choline deficient diet; CDDA diet, choline-deficient L-aminoacid-defined diet; MCS diet, methionine/choline sufficient diet; CSAA diet, choline sufficient L-aminoacid-defined diet; ALT, alanine aminotransferase; Q-PCR, quantitative real-time polymerase chain reaction; TNFα, tumour necrosis factor-α; IL, interleukin; CCL2, C-C-motif chemokine ligand 2; CXCL10, C-X-C-motif chemokine ligand 10; IHC, Immunohistochemistry; VEGF, vascular-endothelial growth factor; TGFβ1, transforming growth factor β1; α-SMA, α-smooth muscle actin.

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ABSTRACT

Mechanisms underlying progression of non-alcoholic fatty liver disease (NAFLD) are still incompletely characterized. Hypoxia and hypoxia inducible factors (HIFs) have been implicated in the pathogenesis of chronic liver diseases but the actual role of HIF-2α in the evolution of NAFLD has never been investigated in detail. In this study, we show that HIF-2α is selectively overexpressed in the cytosol and the nuclei of hepatocytes in a very high percentage (> 90%) of liver biopsies from a cohort of NAFLD patients at different stage of the disease evolution. Similar features were also observed in mice with steatohepatitis induced by feeding a methionine/choline-deficient (MCD) diet. Experiments performed in mice carrying hepatocyte-specific deletion of HIF-2α and related control littermates fed with either choline-deficient L-amino acid-refined (CDAA) or MCD diets showed that HIF-2α deletion ameliorated the evolution of NAFLD by decreasing parenchymal injury, fatty liver, lobular inflammation and the development of liver fibrosis. The improvement in NAFLD progression in HIF-2α deficient mice was related to a selective down-regulation in the hepatocyte production of Histidine-Rich Glycoprotein (HRGP), recently proposed to sustain macrophage M1 polarization. In vitro experiments confirmed that the up-regulation of hepatocyte HRGP expression was hypoxia- and HIF-2α-dependent. Finally, analyses performed on specimens from NAFLD patients indicated that HRGP was overexpressed in all patients showing hepatocyte nuclear staining for HIF-2α and revealed a significant positive correlation between HIF-2α and HRGP liver transcripts levels in these patients.

Conclusions: These results indicate that hepatocyte HIF-2α activation is a key feature in both human and experimental NAFLD and significantly contributes to the disease progression through the up-regulation of HRGP production.

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Non-alcoholic fatty liver disease (NAFLD), regarded as the hepatic manifestation of the metabolic syndrome, is becoming the most frequent chronic liver disease (CLD) worldwide with a prevalence up to 20-30% in the general population and even higher among obese individuals and/or patients affected by Type II diabetes mellitus.[1-6] NAFLD can evolve (20-30% of patients) in non-alcoholic steatohepatitis (NASH), characterized by hepatocyte injury and lobular inflammation that, in turn, can progress to fibrosis, cirrhosis and hepatocellular carcinoma (HCC), but at present mechanisms underlying NAFLD progression are still incompletely characterized and no validated therapy is currently available.[1,4-7]

Literature data suggest that hepatic hypoxia can have a role in CLD progression and HCC development by sustaining both angiogenesis and fibrogenesis as well as, possibly, also inflammatory response and autophagy, with hepatic myofibroblasts being able to both respond to hypoxia and to act in pro-angiogenic way. [8-11] The cellular response to hypoxia is mainly operated by hypoxia-inducible factors (HIFs), evolutionary conserved heterodimeric transcriptional factors consisting of an oxygen-sensitive α-subunit (HIF-1α or HIF-2α) and a stable, constitutively expressed, β-subunit (HIF-1β).[12-14] So far, data concerning the role of hypoxia and HIFs in NAFLD are quite limited [10] but morphological evidence indicate that liver hypoxia develops in parallel with NAFLD-mediated steatosis, particularly in peri-venous areas, similarly to what described for ethanol-induced fatty liver. [15,16] Nonetheless, HIFs can be regulated also by oxygen-independent mechanisms such as mitochondrial dysfunction, reactive oxygen species (ROS) generation and endoplasmic reticulum stress. [8-14] On their turn, HIF-1α and HIF-2α can modulate the cellular adaptive responses to hypoxia by up-regulating either common or, more often, distinct and non-overlapping transcriptional programs. For instance, HIF-1α promotes glucose consumption and glycolysis while lipid storage is mainly regulated by HIF-2α. [12-14] These actions on cell metabolism are potentially relevant for the pathogenesis of NAFLD since hypoxic conditions have been

reported to stimulate lipid storage and inhibit lipid catabolism through β-oxidation.[10,12,13,17] Although both HIF-1α and HIF-2α affect lipid metabolism, experimental studies indicate that HIF-2α activation leads to fatty liver by both up-regulating genes involved in fatty acid synthesis/uptake and lipid storage and down-regulating those involved in fatty acid catabolism.[18,19] HIF-2α activation has also been related to an early increase in the transcription of pro-inflammatory cytokines and of genes involved in fibrogenesis. [19] However, these data have been obtained in mice carrying multiple genetic manipulations in the absence of liver injury [18,19] or in the frame of a short protocol (2 weeks) of ethanol administration using Lieber-De Carli liquid diet, all conditions that do not reproduce the conditions occurring in NAFLD.[19] At present, relevant human data about the role of HIF-2α in fatty liver development and NAFLD progression are lacking. On the other hand, despite HIF-1α appears to sustain fibrogenesis in the bile duct ligation experimental model of CLD, [20] other studies designed to specifically target hepatocyte HIF-1α in experimental models of alcoholic or NAFLD-related steatosis and progression have led to conflicting results. [21-24]

In this study, the analysis of liver biopsies from a cohort of NAFLD patients and the induction of experimental NAFLD with two different dietary protocols in mice carrying hepatocyte-specific deletion of HIF-2α provide evidence indicating that HIF-2α plays a critical role in NAFLD progression by up-regulating hepatocyte expression of histidine-rich glycoprotein (HRGP).

Material & Methods

Human Subjects

The study on NAFLD patients was approved by the ethics' committee of the Azienda Ospedaliera Universitaria Città della Salute, Torino, Italy. For this study we analyzed liver biopsies from NAFLD patients (n=27) ranging from early disease (staged F0-F1) to more advanced conditions of fibrosis (staged F2-F3) or cirrhosis (F4) and referring to the Division of Gastro-Hepatology of the University of Turin. All samples were collected at the time of first diagnosis. Patients were characterized by anthropometric, clinical and biochemical data and liver biopsies were evaluated for the severity of steatohepatitis and fibrosis according to Kleiner et al. [25] All subjects gave informed consent to the analysis and the study protocol, conformed to the ethical guidelines of the 1975 Declaration of Helsinki, was planned according to the guide-lines of the local ethical committee. The clinical and biochemical features of the patients are reported in Supplementary Table 1. Control human liver tissue (n=5), as defined for normal histological structures in hematoxylin and eosin (H&E) sections and the absence of inflammation in the portal tract and parenchyma, was obtained from diagnostic biopsies or from resection samples taken at a distance of more than 5 cm from the border of liver metastasis of colon carcinoma.

Animal experimentation

Mice carrying a hepatocyte-specific deletion of HIF-2α (hHIF-2α $^{-/-}$) were obtained by breeding HIF-2α $^{fl/fl}$ C57BL/6 mice with mice on the same genetic background expressing the Cre-recombinase under the control of the Albumin promoter (Alb/Cre $^{+/+}$ mice) (Jackson Laboratories, Bar Harbor, Maine, USA). Eight weeks old male hHIF-2α $^{-/-}$ mice and related control sibling littermates not carrying HIF-2α deletion, were fed with either methionine/choline deficient (MCD) diet for 4 or 8 weeks or choline-deficient L-aminoacid-defined (CDDA) diet (Laboratorio Dottori Piccioni, Gessate,

Italy) for 12 and 24 weeks.[26,27] Control littermates received the corresponding methionine/choline or choline sufficient diets (MCS or CSAA, respectively). In preliminary experiments eight-week-old normal C57BL/6 mice were fed on the MCD diet or to MCS control diet for 4 and 8 weeks. The experiments complied with national ethical guidelines for animal experimentation and the experimental protocols were approved by the Italian Ministry of Health.

Biochemical Analyses.

Plasma alanine aminotransferase (ALT) and liver triglycerides were determined by spectrometric kits supplied by Radim S.p.A. (Pomezia, Italy) and Sigma Diagnostics (Milano, Italy), respectively. Circulating IL-12 was evaluated by commercial ELISA kits supplied by Peprotech (Milano, Italy).

Immunohistochemistry, Sirius Red staining and histo-morphometric analysis.

Immunohistochemistry,[28,29] Picro-Sirius Red staining and histomorphometric analysis [30,31] were performed on paraffin-embedded human liver biopsies and/or murine liver specimens as previously reported. More details are in the Supplementary material.

Quantitative real-time PCR (Q-PCR).

RNA extraction, complementary DNA synthesis, quantitative real-time PCR (Q-PCR) reactions were performed on human and murine liver speciments as previously described. [28-30] mRNA levels were measured by Q-PCR, using the SYBR® green method as described. [28-30] More details and oligonucleotide sequences of primers used for Q-PCR are available in the Supplementary Material section. The murine transcripts for tumour necrosis factor-α (TNFα), CD11b, interleukin (IL) -12p40, C-C-motif chemokine ligand 2 (CCL2), C-X-C-motif chemokine ligand 10 (CXCL10), HRGP, β-actin liver RNA were retro-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy). Q-PCR for these transcripts was performed in a Techne TC-312

thermacycler (TecneInc, Burlington NJ, USA) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for indicated murine genes (Applied Biosystems Italia, Monza, Italy) as previously described. [26,31] Human sample analysis was performed using SsoFast™ EvaGreen® Supermix (Biorad, Hercules, CA, USA) following manufacturer's instructions.

Intrahepatic mononucleated cell isolation and flow cytometry analysis.

Hepatic mononucleated cells were isolated from the livers of naive and CDAA-fed mice and purified on a density gradient (Lympholyte®-M, Cedarlane Laboratoires Ltd. Burlington, Canada) as described. [31] Cells were washed with Hank's medium and incubated 30 min with de-complemented mouse serum to block unspecific immunoglobulin binding. The cells were then stained with fluorochrome-conjugated antibodies for CD45, CD11b, Ly6C, (eBiosciences, San Diego CA, USA), F4-80 (Invitrogen, Abingdon, UK) and analyzed with a AttuneTM NxT acoustic focusing cytometer (Thermo Fischer Scientific, , Waltham, MS, USA) following prior gating for CD45 and the absence of cell aggregates. Intracellular staining for IL-12 was performed using a specific fluorochrome-conjugated antibody (eBiosciences, San Diego CA, USA).

In vitro experiments and Western Blot analysis

In vitro experiments in this study were performed in normal HepG2 cells (American Type Culture Collection, USA) as well as in cells stably transfected in order to overexpress HIF-2α in either normoxic or hypoxic conditions, as previously reported. [28] Western blot analysis was performed on total cell or tissue lysates as previously described. [28,30] More details on in vitro studies and WB analysis are available in Supplementary material.

Data analysis and statistical calculations.

Statistical analyses were performed by SPSS statistical software (SPSS Inc. Chicago IL, USA) using one-way ANOVA test with Tukey's correction for multiple comparisons or Kruskal-Wallis test for non-parametric values. Significance was taken at the 5% level. Normality distribution was preliminary assessed by the Kolmogorov-Smirnov algorithm.

Results

HIF-2α is overexpressed in human and murine hepatocytes in NAFLD-related liver specimens

Immunohistochemistry (IHC) analysis of liver biopsies from a cohort of well characterized NAFLD patients (n= 27, see Supplementary Table 1 for patient characterization) at different stages of the disease evolution showed a well evident increase of HIF-2α immune-staining as compared to undamaged liver samples (n=5) from patients undergoing resection for hepatic metastasis of colon carcinoma. In the majority of NAFLD patients (25 out of 27; 92%) HIF-2α positivity was detectable almost exclusively in hepatocytes (Figure 1A,B) and involved both the cytoplasm and the nuclei, although the number of positive nuclei varied within patients and also in different areas of the same biopsy (Figure 1A). The nuclei of non-parenchymal cells, mainly inflammatory cells or myofibroblast-like cells in fibrotic septa, were essentially negative for HIF-2α. Occasionally, HIF-2α staining was observed in portal/periportal cholangiocytes, particularly in subjects with advanced fibrosis. Semi-quantitative assessment of HIF-2α positive hepatocyte nuclei confirmed a significant increase among NAFLD patients irrespectively from the stage of the disease (Figure 1B). These findings were reproduced in a rodent model of NAFLD-related liver fibrosis based on mice feeding with a methionine and choline-deficient (MCD) diet up to 8 weeks (Supplementary Figure 1A,B). In fact, while in control mice HIF-2α staining was only detected in the cytoplasm of hepatocytes closely surrounding centrilobular veins (Supplementary Figure 1A), extensive HIF-2α positivity was evident in liver sections from mice receiving the MCD diet (Supplementary Figure 1B). In these animals Q-PCR analyses confirmed that both HIF-2α as well HIF-1α transcripts were up-regulated in mice with NAFLD, but only those of HIF-2α paralleled the disease progression (Supplementary Figure 1 C-F).

Hepatocyte-specific deletion of HIF-2α improves NAFLD-associated liver injury, steatosis, inflammatory response and fibrosis

Based on previous data, we seek to evaluate whether HIF-2α deletion in hepatocytes might modify NAFLD evolution. To this aim, progressive NAFLD was induced in mice carrying hepatocyte-specific deletion of HIF-2α (hHIF-2α$^{-/-}$ mice) as well as related control littermates by two dietary protocols using a MCD diet, administered for 4 and 8 weeks and a choline-deficient L-aminoacid-defined (CDDA) diet, administered for 12 and 24 weeks. In preliminary experiments, we first checked for effective HIF-2α depletion in the livers of hHIF-2α$^{-/-}$ mice receiving the MCD diet. As reported in Supplementary Figure 2A-C, Q-PCR analysis revealed a very significant down-regulation of liver HIF-2α transcripts in hHIF-2α$^{-/-}$ mice as compared to control littermates, whereas HIF-1α and vascular-endothelial growth factor (VEGF) transcript levels were not affected. HIF-2α deletion was also confirmed at protein levels by western blotting of total liver proteins (Supplementary Figure 2D).

Interestingly, in hHIF-2α$^{-/-}$ mice receiving the MCD diet for 4 weeks the severity of steatohepatitis as evaluated by histology, alanine aminotransferase (ALT) release and by hepatic triglycerides accumulation was significantly lower than in HIF-2α sufficient animals (Figure 2 A-C). The extent of lobular inflammation and the hepatic expression of inflammatory markers TNF-α, CD11b, and CCL2 were also decreased in hHIF-2α$^{-/-}$ mice receiving the MCD diet (Figure 2D). On the same line, using CDAA fed mice we observed that the deletion of hepatocyte HIF-2α (which was confirmed also by means of IHC in these mice vs control littermates, see Suppl. Figure 3) slowed-down the time-dependent progression of NAFLD (Figure 3A,B,E) without affecting the gain in body weight and the development of insulin resistance (Figure 3C,D). In this experimental model, hHIF-2α$^{-/-}$ mice also showed less liver fibrosis at both 12 and 24 weeks, as evidenced by Sirius Red staining (Figure 4A,B). This paralleled a decrease in hepatic transcript for transforming growth factor β1 (TGFβ1),

α-smooth muscle actin (α-SMA) and collagen 1A1 (Figure 4C), as well as in the number of α-SMA positive myofibroblasts detected by immunohistochemistry (Figure 4D). Remarkably, the protection from fibrosis was also evident in hHIF-2α$^{-/-}$ mice receiving the MCD diet up to 8 weeks as compared to control HIF-2α sufficient littermates (Suppl. Figure 4A-D).

Hepatocyte-specific deletion of HIF-2α prevents the progression of experimental NAFLD by affecting hepatocyte-dependent release of histidine-rich glycoprotein (HRGP)

To address the mechanisms by which hepatocyte HIF-2α up-regulation promotes inflammatory mechanisms in NAFLD we focused our attention on the role of histidine-rich glycoprotein (HRGP), a hepatocyte-released mediator (i.e., hepatokine) that has been recently shown to support liver macrophage activation in experimental and clinical NAFLD/NASH.[32] To investigate the possible hypoxia- and HIF-2α-dependent modulation of HRGP we employed HepG2 cells, which are known to rapidly respond to hypoxia with HIF-2α stabilization and the up-regulation of HIF-2α target genes.[28,33] As shown in Figure 5A the incubation of HepG2 cells under hypoxic condition, which is known to rapidly promote the nuclear translocation of HIF-2α,[28] was associated to an appreciable increase in HRGP synthesis. A very significant up-regulation of HRGP transcription was also observed in HepG2 transfected with an HIF-2α-containing, but not with an empty vector (Figure 5B), confirming a HIF-2α-dependent modulation of HRGP in liver cells. According with the in vitro data, hepatic HRGP transcript levels increased in a time-dependent manner in control mice fed with CDAA diet in parallel with the upregulation of the expression of another HIF-2α-dependent gene such as CXCR4 (Figure 5C). Conversely, HRGP up-regulation associated with NAFLD evolution was almost completely prevented at both mRNA and protein level in hHIF-2α$^{-/-}$ mice (Figure 5C,D). The relationships between HIF-2α and HRGP were further confirmed by the evidence that HepG2 stably transfected to overexpress HIF-2α also overexpressed transcript levels

of other HIF-2α target genes such as CXCR4 and erythropoietin (Suppl. Figure 5 A,B) or, as previously reported, SerpinB3. [28] Moreover, HRGP transcript and protein levels were down-regulated in HepG2 exposed to hypoxic conditions following efficient silencing for HIF-2α (Suppl. Figure 5 C,D). Immunohistochemistry performed on liver from mice fed with the CDAA diet confirmed that HRGP was selectively expressed by hepatocytes of control mice with steatohepatitis, but not in those from hHIF-2α$^{-/-}$ animals (Figure 5E). HIF-2α and HRPG up-regulation were unrelated to choline deficiency and were also observed in mice with NAFLD induced by administration in the drinking water of a high fat / fructose (HF/F) diet (Suppl. Figure 6 A-D). Hepatocyte-derived HRGP has been recently shown to promote liver inflammation by stimulating M1 polarization of hepatic macrophages.[32] In our hands, the impaired production of HRPG in mice receiving the CDAA diet decreased the liver infiltration by F4/80 positive macrophages as evidence by both immunohistochemistry (Figure 6A) and flow cytometry (Suppl. Fig 7A). Furthermore, the fraction of inflammatory Ly6C^{high} hepatic macrophages and their capacity to produce IL-12 was significantly lower in hHIF-2α$^{-/-}$ mice as compared to control littermates (Figure 6B). Consistently the hepatic expression of M1 cyto/chemokines TNFα, IL-12p40, CCL2 and CXCL10 (Suppl Figure 7B) and the serum levels of IL-12 were also significantly reduced in hHIF-2α$^{-/-}$ mice receiving the CDAA diet (Figure 6C) indicating a possible role of HRGP in the mechanisms by which the stimulation of HIF-2α in parenchymal cells supports pro-inflammatory responses of hepatic macrophages.

Finally, to further support the possible relevance of the relationships between HIF-2α and HRGP in the human disease, we analyzed for HIF-2α and HRGP immune-staining serial sections (4 μm thick) in our cohorts of NAFLD patients. Figure 7A shows that HRGP positivity was very low in sections from control livers. Conversely, in agreement with previous data,[32] HPRG immune-staining was well evident and increased in liver sample from NAFLD patients irrespectively from the disease

evolution (Figure 7B,C) and co-localized with that of HIF-2α (Figure 7A). Furthermore, we found a significant positive correlation between HIF-2α and HRGP mRNA levels in those patients of this cohort (n=19) for whom frozen liver specimens were available (Figure 7D).

Discussion

As mentioned in the introduction, hypoxia and HIFs have been proposed to play a role in the progression of CLD. [8-11] However, data concerning their role in the evolution of NAFLD are quite limited [10] and, more specifically, the contribution of HIF-2α has never been investigated in detail. In the present study we provide evidence that hepatocyte HIF-2α is up-regulated in either human or experimental NAFLD and we propose a mechanism by which HIF-2α activation in the liver parenchyma can contribute to the disease progression.

To our knowledge, the present study is the first to report that HIF-2α is specifically overexpressed in hepatocytes during the development of human NAFLD. In the majority of the liver biopsies from NAFLD patients a nuclear localization of HIF-2α is also evident, indicating that, by forming heterodimer with either HIF-1β or HIF-2β, HIF-2α can act as a transcription factor. Interestingly, HIF-2α activation is evident already in the early stage of the disease (F0-F1) and it is maintained up to more advanced (F3-F4) conditions of fibrosis/cirrhosis. Similar findings have been detected in murine models of NAFLD. In this setting, both HIF-2α and HIF-1α transcripts were up-regulated in mice with steatohepatitis, but only those of HIF-2α paralleled the disease progression. Altogether, these data suggest that HIF-dependent responses are an early event in NAFLD evolution and can be involved in the disease progression. Hepatic hypoxia likely represents the most obvious cause for HIF-2α up-regulation in both human and experimental NAFLD. In fact,

diffuse lobular staining with the hypoxia-sensitive dye pimonidazole has recently documented in mice with NAFLD. [21] Nevertheless, we cannot exclude that hypoxia-independent mechanisms, as for instance oxidative stress, might also contribute to stimulate HIF-2α activity. [8-11]

The actual relevance of HIF-2α in NAFLD progression has been mechanistically confirmed by inducing NAFLD with MCD or CDAA diets in mice carrying a hepatocyte specific deletion of HIF-2α (hHIF-2α$^{-/-}$ mice). We have observed that steatosis, parenchymal injury, inflammatory response and liver fibrosis are appreciably attenuated in these animals, as compared with control littermates. The decrease in fatty liver detected in hHIF-2α$^{-/-}$ mice is consistent with the observation that HIF-2α activation is *per se* capable to promote fatty liver by up-regulating genes involved in fatty acid synthesis/uptake and lipid storage and by down-regulating those involved in fatty acid catabolism. [18,19] Accordingly, in the CDAA diet model transcript levels of SREBP1 and of FASN (Fatty Acid Synthase) were significantly reduced in hHIF-2α-/- mice vs related control littermates exposed to the same diet (Suppl. Figure 8). Moreover, by employing murine hepatocytes (AML12 line), we found that exposure of these cells to palmitic acid (i.e, a condition leading to lipid accumulation and mimicking “in vitro” lipotoxicity) resulted in a significant up-regulation of both HIF-2α and HRGP protein levels (Suppl. Figure 9A) and of HRGP transcript levels (Suppl. Figure 9B), suggesting that also lipotoxicity may represent an additional condition leading to HIF-2α and HRGP up-regulation.

Furthermore, in a previous study using mice fed with a high fat diet the hepatocyte deletion of HIF-1α effectively prevented NAFLD associated liver fibrosis without interfering with fat accumulation. [21] Interestingly, the same work also showed that hepatocyte HIF-1α deficiency moderately affected parenchymal damage and did not influenced the extent of lobular inflammation. [21] This indicates that beside the action on steatosis, the activation of HIF-2α in hepatocytes has a key role in favoring NAFLD-associated cell injury and inflammatory responses.

To investigate whether hepatocyte HIF-2α might stimulate the production of mediators with pro-inflammatory action, we focused the attention on the possible role of HRGP, a multifunctional hepatocyte-derived circulating protein, [34] that has been recently shown to be involved in modulating pro-inflammatory activity of macrophages in tumors as well during chronic liver injury. [32,35] In particular, Bartneck et al. [32] have reported that HRGP deletion leads to a significant protection from liver injury and fibrosis in mice with MCD-induced NASH by down-modulating the recruitment of hepatic macrophages, and their M1 polarization. In this setting, our data demonstrate an interplay between HIF-2α and HRGP during NAFLD evolution by showing that the lack of hepatocyte HIF-2α prevents HRGP up-regulation in mice fed with CDAA diet. Furthermore, HRGP staining of liver biopsies from NAFLD patients co-localizes with that of HIF-2α, while in the same subjects a positive linear correlation is evident between the HIF-2α and HRGP transcripts. These findings are supported by in vitro experiments employing HepG2 cells exposed to hypoxia or overexpressing HIF-2α that confirm the strict HIF-2α dependence of HRGP production.

In line with the view that HIF-2α deficiency can reduce hepatic inflammation by preventing HRGP-mediated support to M1 activation of liver macrophages, we have observed that the liver expression of M1 markers TNF-α, IL-12and CXCL10 is blunted in hHIF-2α$^{-/-}$ mice with NAFLD. Moreover, flow cytometry reveals that the prevalence of pro-inflammatory Ly6C^{high} hepatic macrophages and their capacity to produce IL-12 are lowered in hHIF-2α$^{-/-}$ mice fed the CDAA diet. HIF-2α influence on macrophage responses is of potential general relevance since it is well known that the polarization of hepatic macrophages is a crucial determinant for the progression of CLDs. [36] In particular, macrophage M1 shift has been reported to correlate with the severity of both alcoholic and non-alcoholic liver disease. [37,38] Accordingly, M2 skewing is associated with a more favorable histology and fewer hepatic lesions in patients with ethanol-related CLD [37] as well as in HRGP$^{-/-}$ mice exposed to chronic liver injury. [32]

The involvement of HRGP in mediating the effects of HIF-2α activation during NAFLD progression does not rule out the possible involvement of other mediators. We have previously reported that HIF-2α activation in human liver cancer cells up-regulates Serpin B3 and this latter contributes to the development of fibrosis during CLD by sustaining the most relevant profibrogenic responses by activated stellated cells.[28,30] Unfortunately, at difference of humans, SerpinB3 is virtually undetectable in murine livers during CLD, thus in the present study we did not have the possibility to evaluate its contribution to NAFLD progression. However, based on the association between circulating levels of Serpin B3 and the risk of fibrosis in patients with chronic hepatitis C,[39] a possible role of Serpin B3 in the evolution of human NAFLD cannot be excluded.

In conclusion, data presented indicate that hepatocyte HIF-2α activation during the evolution of human and murine NAFLD has a role in the disease progression by mediating the up-regulation of HRGP expression that, in turn critically influence the severity of steatohepatitis and fibrogenesis. These findings point HIF-2α and HIF-2α-dependent genes as putative targets for future therapeutic strategies in NAFLD, a disease that, at present, has no validated therapy.

REFERENCES

1) Satapathy SK, Sanyal AJ. Epidemiology and Natural History of Nonalcoholic Fatty Liver Disease. Semin Liver Dis 2015;35:221-235.

2. Younossi ZM, Koenig AB, Abdelatif D, Fazel Y, Henry L, Wymer M. Global epidemiology of non-alcoholic fatty liver disease - Meta-analytic assessment of prevalence, incidence and outcomes. Hepatology 2016;64:73-84.

3. Yki-Jarvinen H. Non-alcoholic fatty liver disease as a cause and a consequence of metabolic syndrome. Lancet Diabetes Endocrinol 2014;2:901-910

4. McPherson S, Hardy T, Henderson E, Burt AD, Day CP, Anstee QM. Evidence of NAFLD progression from steatosis to fibrosing-steatohepatitis using paired biopsies: Implications for prognosis and clinical management. J Hepatol 2015;62:1148-1155.

5. Singh S, Allen AM, Wang Z, Prokop LJ, Murad MH, Loomba R. Fibrosis progression in nonalcoholic fatty liver vs nonalcoholic steatohepatitis: a systematic review and meta-analysis of paired-biopsy studies. Clin Gastroenterol Hepatol 2015;13:643–54.e9.

6. Adams LA, Anstee QM, Tilg H, Targher G. Non-alcoholic fatty liver disease and its relationship with cardiovascular disease and other extrahepatic diseases. Gut 2017;66:1138-1153.

7. Torres DM, Harrison SA. Nonalcoholic steatohepatitis and noncirrhotic hepatocellular carcinoma: fertile soil. Semin Liver Dis 2012;32:30-8.

8. Nath B, Szabo G. Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. Hepatology 2012;55:622-633.

9. Wilson GK, Tennant DA, McKeating JA. Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. J Hepatol. 2014 ;61:1397-1406.

10. Lefere S, Van Steenkiste C, Verhelst X, Van Vlierberghe H, Devisscher L, Geerts A. Hypoxia - regulated mechanisms in the pathogenesis of obesity and non-alcoholic fatty liver disease. Cell. Mol Life Sci 2016; 73:3419-3431.

11. Novo E, Cannito S, Paternostro C, Bocca C, Miglietta A, Parola M. Cellular and molecular mechanisms in liver fibrogenesis. Arch Biochem Biophys 2014;548:20-37.

12. Semenza GL. Oxygen sensing, hypoxia-inducible factors, and disease pathophysiology. Annu Rev Pathol 2014;9:47–71.

13. Shay JE, Simon MC. Hypoxia-inducible factors: crosstalk between inflammation and metabolism. Semin Cell Dev Biol 2012;23:389-394.

14. **Majmundar AJ**, **Wong WJ**, Simon MC. Hypoxia-inducible factors and the response to hypoxic stress. Mol Cell 2010;40:294-309.

15. Arteel GE, Iimuro Y, Yin M, Raleigh JA, Thurman RG. Chronic enteral ethanol treatment causes hypoxia in rat liver tissue in vivo. Hepatology 1997;25:920–926.

16. Mantena SK, Vaughn DP, Andringa KK, Eccleston HB, King AL, Abrams GA, et al. High fat diet induces dysregulation of hepatic oxygen gradients and mitochondrial function in vivo. Biochem J 2009;417:183–193.

17. Nakazawa MS, Keith B, Simon MC. Oxygen availability and metabolic adaptations. Nat Rev Cancer 2016;16:663-73.

18. Rankin EB, Rha J, Selak MA, Unger TL, Keith B, Liu Q et al. Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. Mol Cell Biol 2009;29:4527-4538.

19. Qu A, Taylor M, Xue X, Matsubara T, Metzger D, Chambon P, et al. Hypoxia-inducible transcription factor 2α promotes steatohepatitis through augmenting lipid accumulation, inflammation, and fibrosis. Hepatology 2011;54:472-483.

20. Moon JO, Welch TP, Gonzalez FJ, Copple BL. Reduced liver fibrosis in hypoxia-inducible factor-1a-deficient mice. Am J Physiol Gastrointest Liver Physiol 2009; 296:G582–G592.

21. Nath B, Levin I, Csak T, Petrasek J, Mueller C, Kodys K, et al. Hepatocyte-specific hypoxia-inducible factor-1α is a determinant of lipid accumulation and liver injury in alcohol-induced steatosis in mice. Hepatology 2011;53:1526-1537.

22. Mesarwi OA, Shin MK, Bevans-Fonti S, Schlesinger C, Shaw J, Polotsky VY. Hepatocyte Hypoxia Inducible Factor-1 Mediates the Development of Liver Fibrosis in a Mouse Model of Nonalcoholic Fatty Liver Disease. PLoS One. 2016;11:e0168572.

23. Nishiyama Y, Goda N, Kanai M, Niwa D, Osanai K, Yamamoto Y, et al. HIF-1α induction suppresses excessive lipid accumulation in alcoholic fatty liver in mice. J Hepatol 2012; 56:441–447.

24. Ochiai D, Goda N, Hishiki T, Kanai M, Senoo-Matsuda N, Soga T, et al. Disruption of HIF-1α in hepatocytes impairs glucose metabolism in diet-induced obesity mice. Biochem Biophys Res Commun 2011;415:445–449.

25. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

26. Locatelli I, Sutti S, Jindal A, Vacchiano M, Bozzola C, Reutelingsperger C, et al. Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. Hepatology 2015; 60:531-544.

27. De Minicis S, Agostinelli L, Rychlicki C, Sorice GP, Saccomanno S, Candelaresi C, et al. HCC development is associated to peripheral insulin resistance in a mouse model of NASH. Plos One 2014; 9(5):e97136.

28. **Cannito S**, **Turato C**, Paternostro C, Biasiolo A, Colombatto S, Cambieri I, et al. Hypoxia up-regulates SERPINB3 through HIF-2α in human liver cancer cells. Oncotarget 2015; 6:2206-2221.

29. **Novo E**, **Busletta C**, Valfrè di Bonzo L, Povero D, Paternostro C, Mareschi K, et al. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. J Hepatol 2011;54:964-974.

30. **Novo E**, **Villano G**, Turato C, Cannito S, Paternostro C, Busletta C, et al. SerpinB3 promotes pro-fibrogenic responses in activated hepatic stellate cells. Scientific Reports 2017;7(1):3420.

31. Sutti S, Jindal A, Locatelli I, Vacchiano M, Gigliotti L, Bozzola C, et al. Adaptive immune responses triggered by oxidative stress contribute to hepatic inflammation in NASH. Hepatology 2014;59:886-897.

32. **Bartneck M**, **Fech V**, Ehling J, Govaere O, Warzecha KT, Hittatiya K, et al. Histidine-rich glycoprotein promotes macrophage activation and inflammation in chronic liver disease. Hepatology 2016;63:1310-1324.

33. **Turato C**, **Cannito S**, Simonato D, Villano G, Morello E, Terrin L, et al. SerpinB3 and Yap Interplay Increases Myc Oncogenic Activity. Sci Rep 2016; 5:17701.

34. Hulett MD, Parish CR. Murine histidine-rich glycoprotein: cloning, characterization and cellular origin. Immunol Cell Biol 2000;78:280-287.

35. **Rolny C**, **Mazzone M**, Tugues S, Laoui D, Johansson I, Coulon C, et al. HRG inhibits tumor growth and metastasis by inducing macrophage polarization and vessel normalization through downregulation of PlGF. Cancer Cell 2011;19:31-44.

36. Tacke F, Zimmermann HW. Macrophage heterogeneity in liver injury and fibrosis. J Hepatol 2014;60:1090-1096.

37. **Wan J**, **Benkdane M**, Teixeira-Clerc F, Bonnafous S, Louvet A, Lafdil F, et al. M2 Kupffer cells promote M1 Kupffer cell apoptosis: a protective mechanism against alcoholic and nonalcoholic fatty liver disease. Hepatology 2014;59:130-142.

38. Wan J, Benkdane M, Alons E, Lotersztajn S, Pavoine C. M2 kupffer cells promote hepatocyte senescence: an IL-6-dependent protective mechanism against alcoholic liver disease. Am J Pathol 2014;184:1763-1772.

39. Biasiolo A, Chemello L, Quarta S, Cavalletto L, Bortolotti F, Caberlotto C, et al. Monitoring SCCA-IgM complexes in serum predicts liver disease progression in patients with chronic hepatitis. J Viral Hep 2008;15: 246-249.

Author names in bold designate shared co-first authorship.

Figure Legends

Figure 1. **Expression of HIF-2α in human liver disease.** Immunohistochemistry analysis of HIF-2α in paraffin-embedded human liver specimens from NAFLD patients (n = 27) with different degrees of liver fibrosis (F0-F4) (**A**). Control liver refer to surgical resections for hepatic metastasis of colon carcinoma (magnification 20X, 40X, 100X). HIF-2α expression was semi-quantitatively scored blinded, by a pathologist. $P < 0.05$ (Mann-Whitney's U test). (**B**).

Figure 2. Liver injury and inflammatory markers in hHIF2α $^{-/-}$ and control mice with NAFLD induced by feeding a MCD diet. hHIF2α$^{-/-}$ and wild type littermates (LM) were fed a MCD diet for 4 weeks and the following parameters were evaluated: **(A-C)** Parenchymal injury and liver steatosis, as evaluated by measuring **(A)** serum levels of alanine (ALT)**, (B)** hepatic TG content **or,** morphologically, by hematoxylin/eosin staining (magnification 10X) **(C)**; **(D)** Liver expression of inflammatory markers TNF-α, CD11b and CCL2, as evaluated by quantitative real-time PCR (Q-PCR). The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression and are means ± SD of 6-8 animals per group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 3. **Liver injury in hHIF2α$^{-/-}$ and control mice fed with a CDAA diet**. hHIF2α$^{-/-}$ and wild type littermates (LM) were fed CDAA diet for 12 or 24 weeks and the following parameters were evaluated: Parenchymal injury, as evaluated by measuring the serum levels of alanine (ALT) **(A)** or hepatic TG content **(B);** Body weight change **(C)** and plasma glucose concentration (**D**). Liver morphology in mice exposed to CDAA diet was evaluated by hematoxylin/eosin staining

(magnification 10X) (**E**). The values are means ± SD and refer to 6-8 animals per group. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 4. **Liver fibrosis "in vivo" in hHIF-2α$^{-/-}$ mice vs LM mice fed with CDAA diet**. Liver fibrosis was evaluated morphologically in hHIF-2α$^{-/-}$ mice and wild type littermates (LM) mice following 12 or 24 weeks on the CDAA or control CSAA diets by Sirius red staining (**A**) and immunohistochemistry for α-SMA (**D**). Original magnification as indicated. ImageJ software analysis was performed for Sirius red staining to evaluate the amount of fibrosis **(B)**. Analysis by quantitative real-time PCR (Q-PCR) of transcript levels of pro-fibrogenic genes collagen 1A1, αSMA, TGFβ1 in the different experimental groups (**C**). The values are means ± SD and refer to 6-8 animals per group. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 5. Modulatory effect of HIF2α on HRGP. Western blotting analysis of HIF2α and HRGP levels in HepG2 cells exposed to hypoxic conditions (**A**). Q-PCR analysis of HRGP transcript in control HepG2 cells (HepG2 C: not transfected; EV: transfected with empty vector) or HepG2 cells stably transfected to over-express HIF-2α (**B**). Data in graphs are expressed as means ± SEM. qPCR analysis of HRGP and CXCR4 transcripts in wild type littermates (LM) or hHIF-2α$^{-/-}$ mice fed with the control diet (CSAA) or with the CDAA diet for 12 and 24 weeks (**C**). Hepatic mRNAs was as fold increase over control values after normalization to the β-actin gene expression. The values are means ± SD and refer to 6-8 animals per group. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons). Western blotting analysis of HRGP levels in liver of hHIF-2α$^{-/-}$ and littermate mice fed with CDAA diet for 24 weeks (**D**). Histograms report densitometric analyses normalized for the relative GAPDH content. Localization of HRGP expression by IHC in the livers of wild type littermates (LM) or hHIF-2α$^{-/-}$ CDAA or CSAA fed

animals. Original magnification as indicated. ImageJ software analysis was performed for quantification of HRGP staining (**E**). Data are expressed as means ± SD.

Figure 6. Inflammatory markers in hHIF-2α$^{-/-}$ and control mice fed with a CDAA diet. hHIF-2α$^{-/-}$ and wild type littermates (LM) mice were fed with a CDAA diet or CSAA diet for 12 or 24 weeks and the detection of macrophages positive for F4/80 was evaluated by IHC (original magnification as indicated). ImageJ software analysis was performed for quantification of F4/80 staining (**A**). Data are expressed as means ± SD. Hepatic myeloid cells were isolated from livers of either hHIF-2α$^{-/-}$ mice and wild type littermates (LM) fed with the CDAA diet analyzed by flow cytometry for Ly6C and IL expression in Cd11b/F4-80-positive liver macrophages. (**B**) Data are expressed as means ± SD. Circulating IL-12 levels were determined by ELISA assay (**C**). Values refer to 6-8-10 animals per group; boxes include the values within 25th and 75th percentile, whereas horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

Figure 7. HRGP expression in human livers with NASH. HRGP detection by IHC in liver specimens from NASH patients (n =27) with different degrees of liver fibrosis (F0-F4, NAS 2-6). Control liver sample refer to surgical resections for hepatic metastasis of colon carcinoma. Original magnification as indicated. ImageJ software analysis was performed for quantification of HRGP staining (**A**). Data are expressed as means ± SD. HRGP expression was semi-quantitatively scored by a pathologist. P value were calculated with Mann-Whitney's U test (**B**). Relationship between HRGP and HIF2α mRNA in human NASH patients (F0-F4, NAS 2-6) (**C**). The values represent the relative mRNA content. The correlation analysis was performed with Spearman r test.

Figure 1

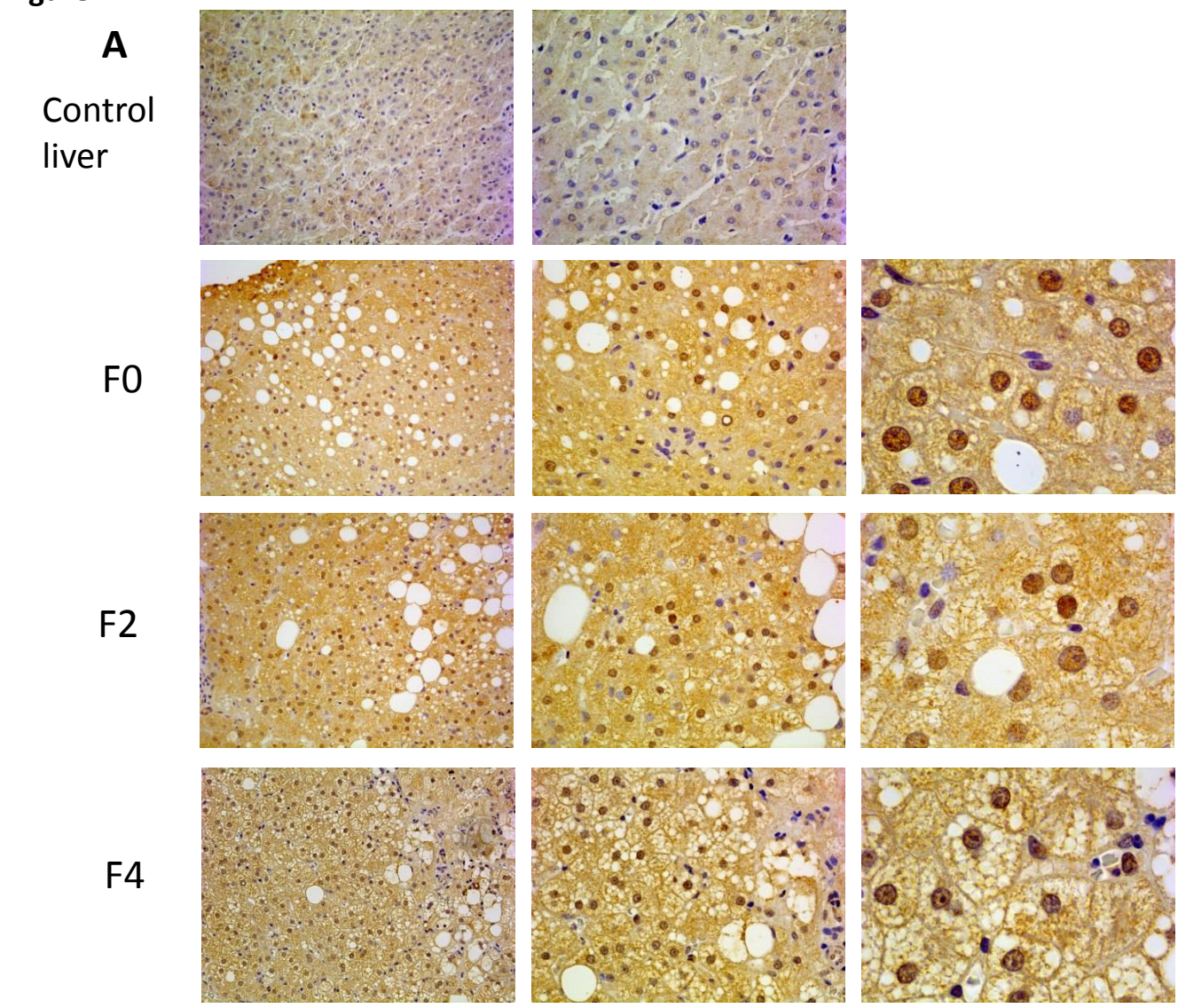

HIF2α expression in NAFLD/NASH patients
A
Control liver
F0
F2
F4
B
HIF-2α nuclear staining
p<0.05
p<0.05
p<0.05
IHC scoring
0
1
2
3
Control
F0-F1
F2
F3-F4
Fibrosis score

Figure 2

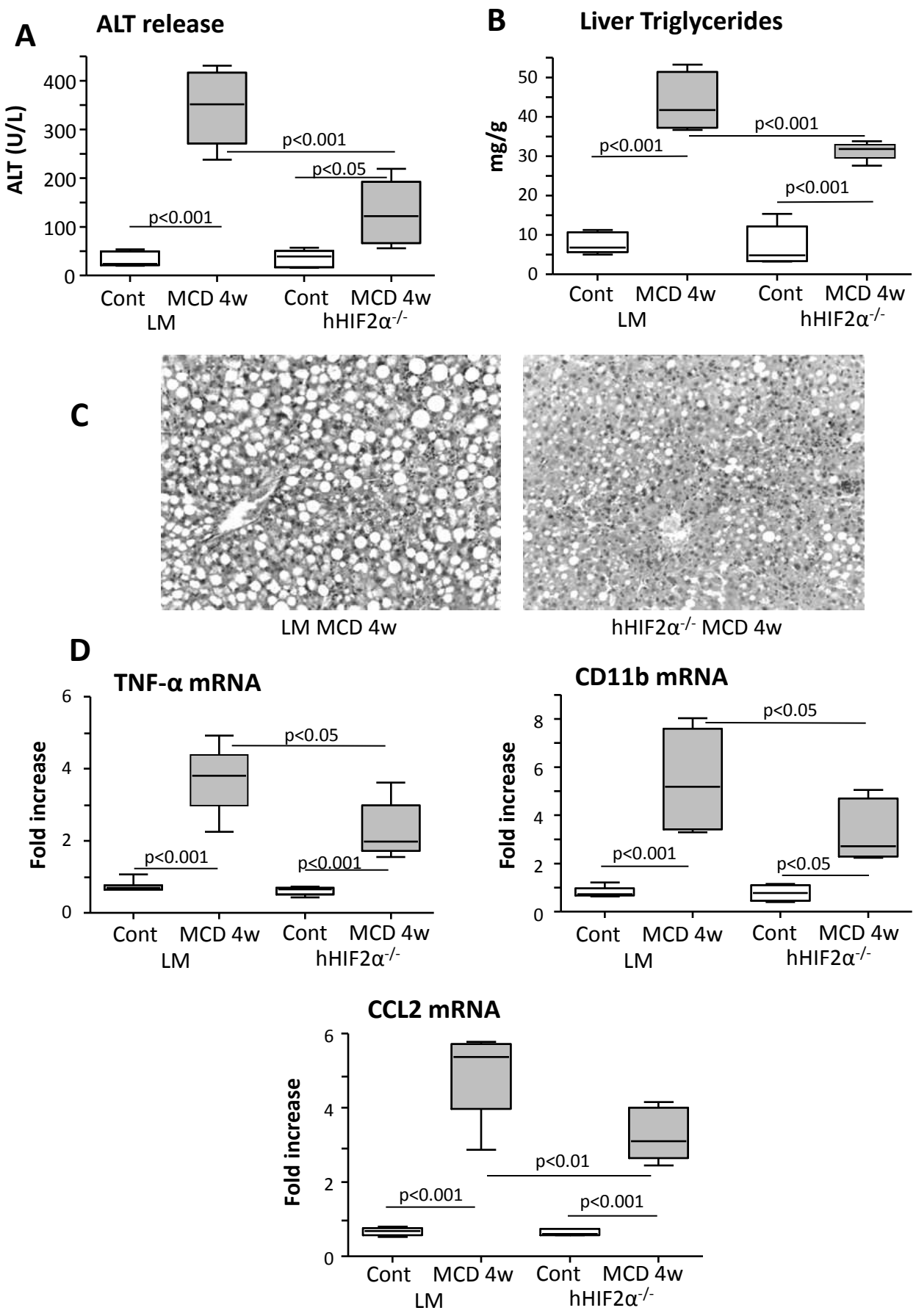

A
ALT release
ALT (U/L)
400
300
200
100
0
p<0.001
p<0.05
p<0.001
Cont
MCD 4w
Cont
MCD 4w
LM
hHIF2α-/-
B
Liver Triglycerides
mg/g
50
40
30
20
10
0
p<0.001
p<0.001
p<0.001
Cont
MCD 4w
Cont
MCD 4w
LM
hHIF2α-/-
C
LM MCD 4w
hHIF2α-/- MCD 4w
D
TNF-α mRNA
Fold increase
6
4
2
0
p<0.05
p<0.001
p<0.001
Cont
MCD 4w
Cont
MCD 4w
LM
hHIF2α-/-
CD11b mRNA
Fold increase
8
6
4
2
0
p<0.05
p<0.001
p<0.05
Cont
MCD 4w
Cont
MCD 4w
LM
hHIF2α-/-
CCL2 mRNA
Fold increase
6
4
2
0
p<0.01
p<0.001
p<0.001
Cont
MCD 4w
Cont
MCD 4w
LM
hHIF2α-/-

Figure 3

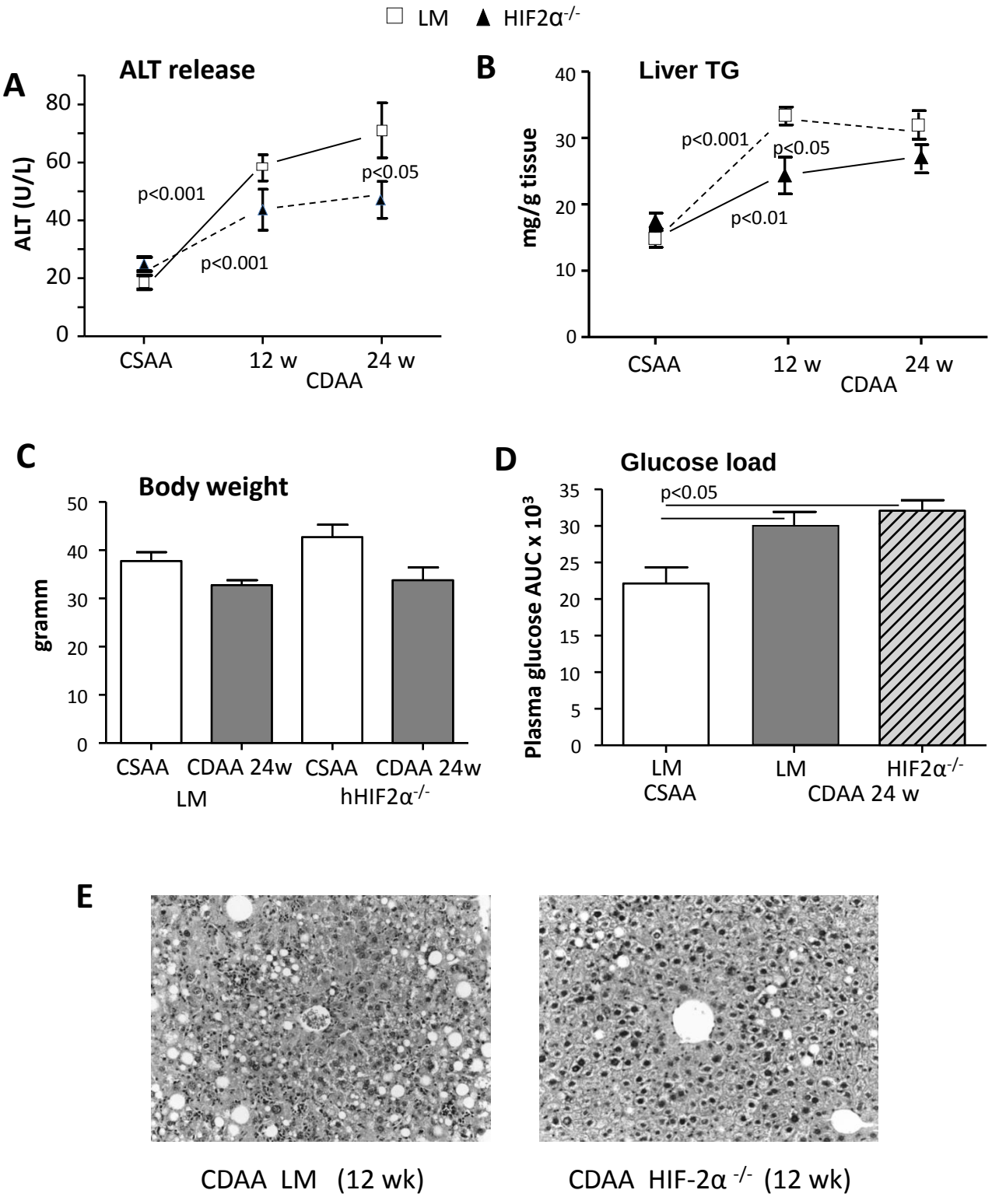

LM
HIF2α-/-
A
ALT release
ALT (U/L)
80
60
40
20
0
p<0.001
p<0.001
p<0.05
CSAA
12 w
24 w
CDAA
B
Liver TG
mg/g tissue
40
30
20
10
0
p<0.001
p<0.05
p<0.01
CSAA
12 w
24 w
CDAA
C
Body weight
gramm
50
40
30
20
10
0
CSAA
CDAA 24w
CSAA
CDAA 24w
LM
hHIF2α-/-
D
Glucose load
Plasma glucose AUC x 10³
35
30
25
20
15
10
5
0
p<0.05
LM
LM
HIF2α-/-
CSAA
CDAA 24 w
E
CDAA LM (12 wk)
CDAA HIF-2α -/- (12 wk)

Figure 4

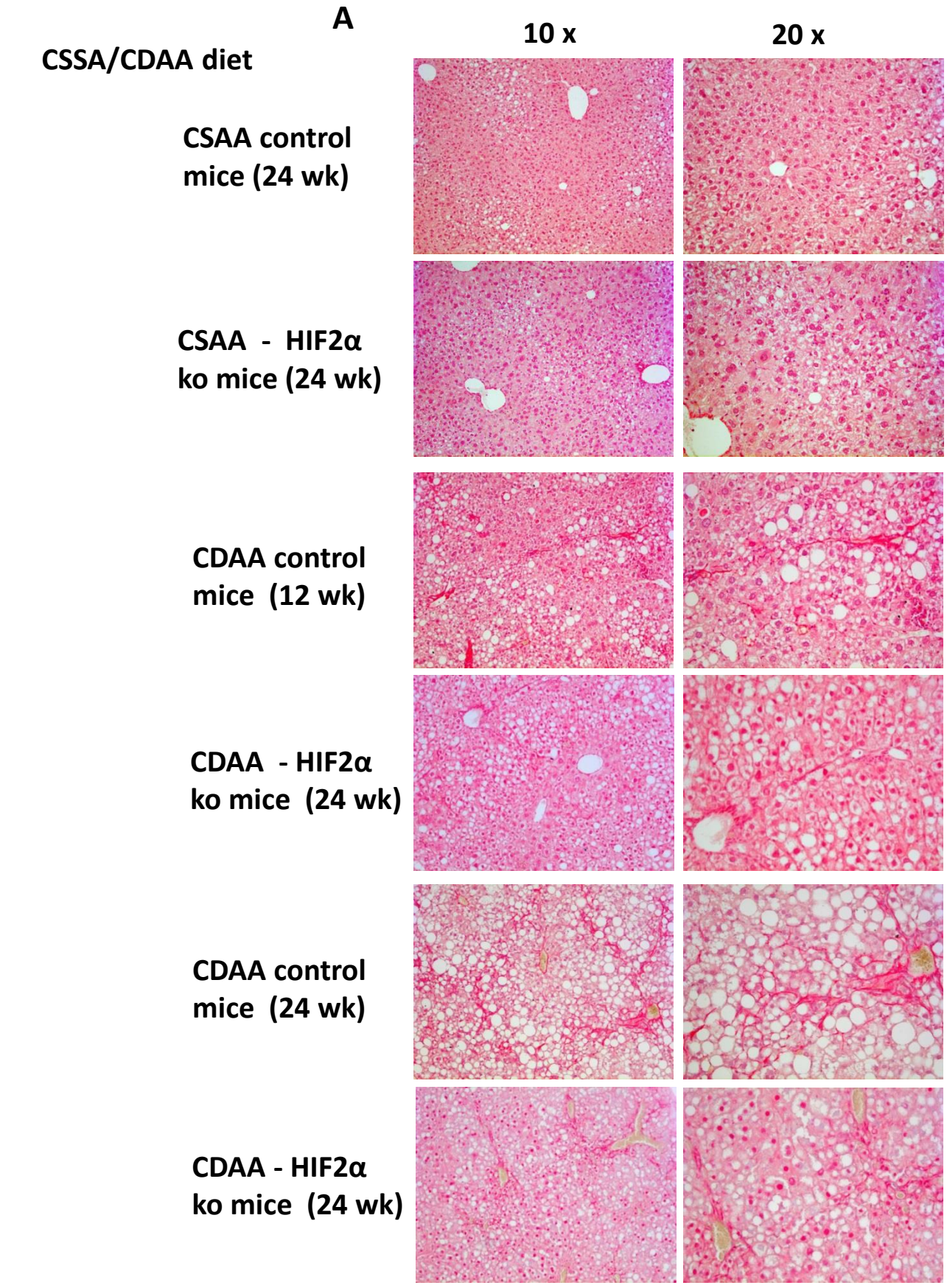

A
10 x
20 x
CSSA/CDAA diet
CSAA control mice (24 wk)
CSAA - HIF2α ko mice (24 wk)
CDAA control mice (12 wk)
CDAA - HIF2α ko mice (24 wk)
CDAA control mice (24 wk)
CDAA - HIF2α ko mice (24 wk)

Figure 4

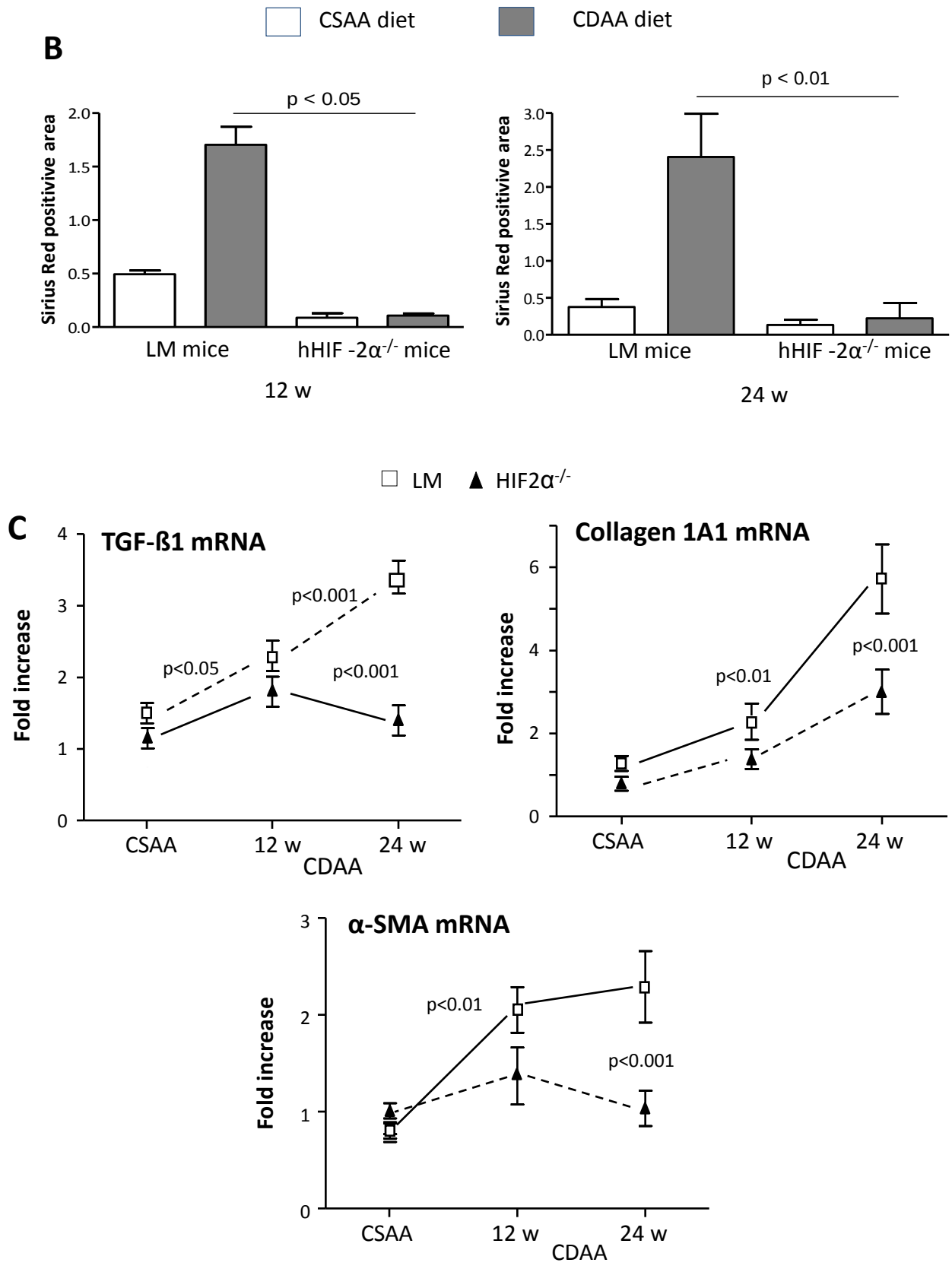

CSAA diet
CDAA diet
B
p < 0.05
Sirius Red positivive area
LM mice
hHIF -2α-/- mice
12 w
p < 0.01
Sirius Red positivive area
LM mice
hHIF -2α-/- mice
24 w
LM
HIF2α-/-
C
TGF-ß1 mRNA
Fold increase
p<0.05
p<0.001
p<0.001
CSAA
12 w
24 w
CDAA
Collagen 1A1 mRNA
Fold increase
p<0.01
p<0.001
CSAA
12 w
24 w
CDAA
α-SMA mRNA
Fold increase
p<0.01
p<0.001
CSAA
12 w
24 w
CDAA

Figure 4

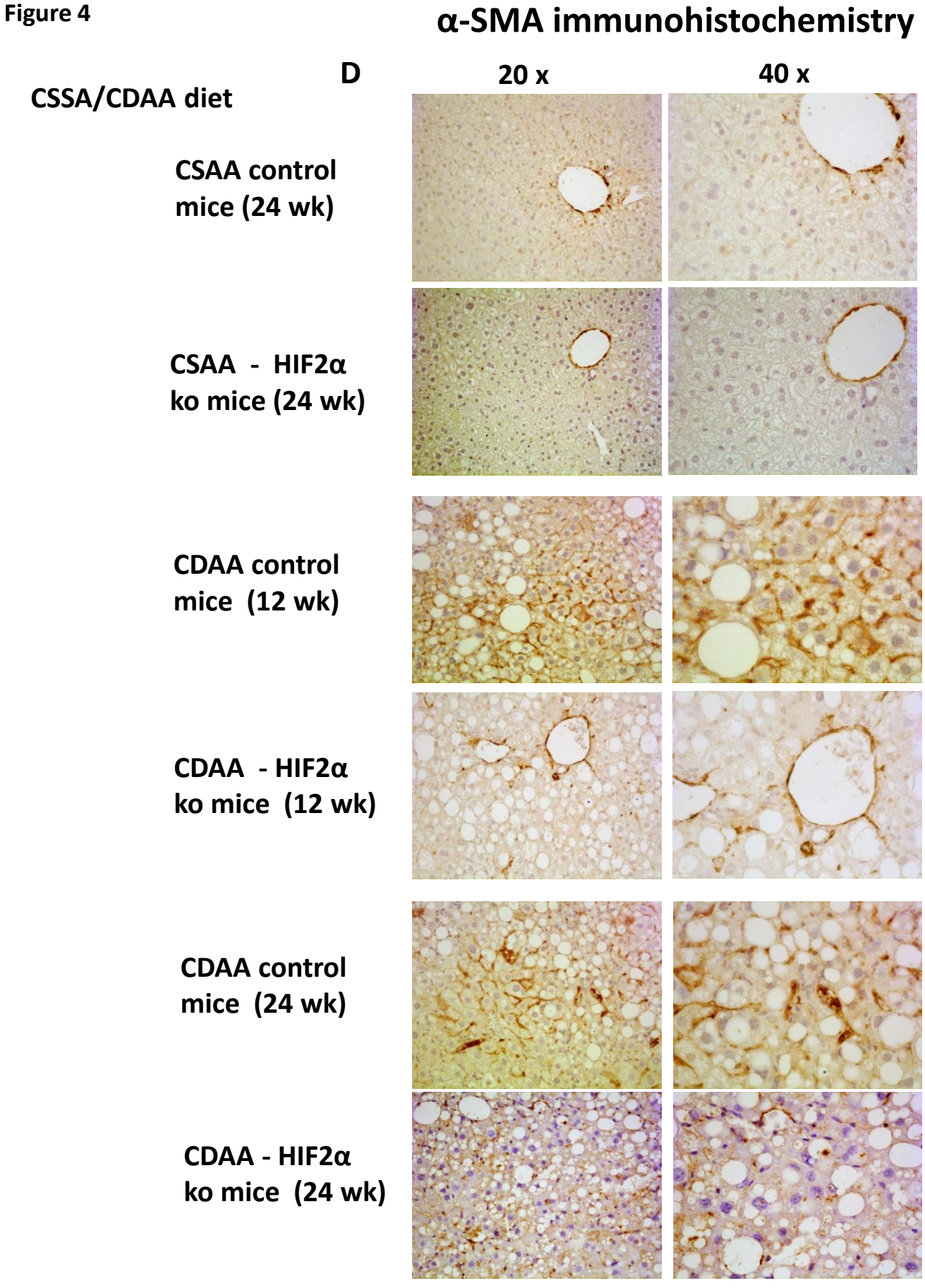
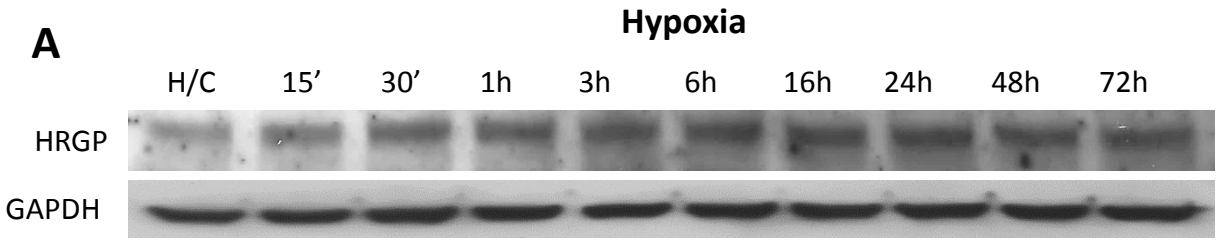
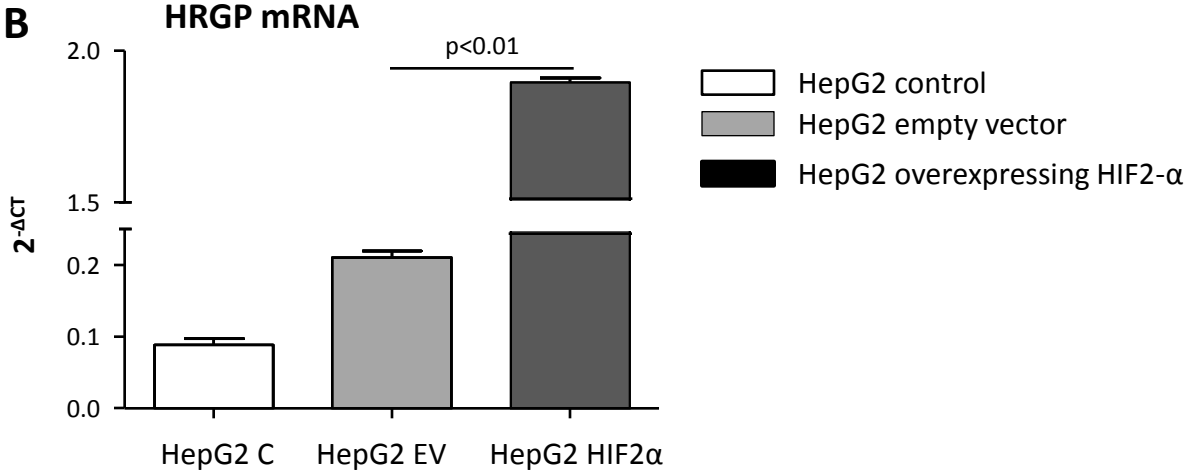
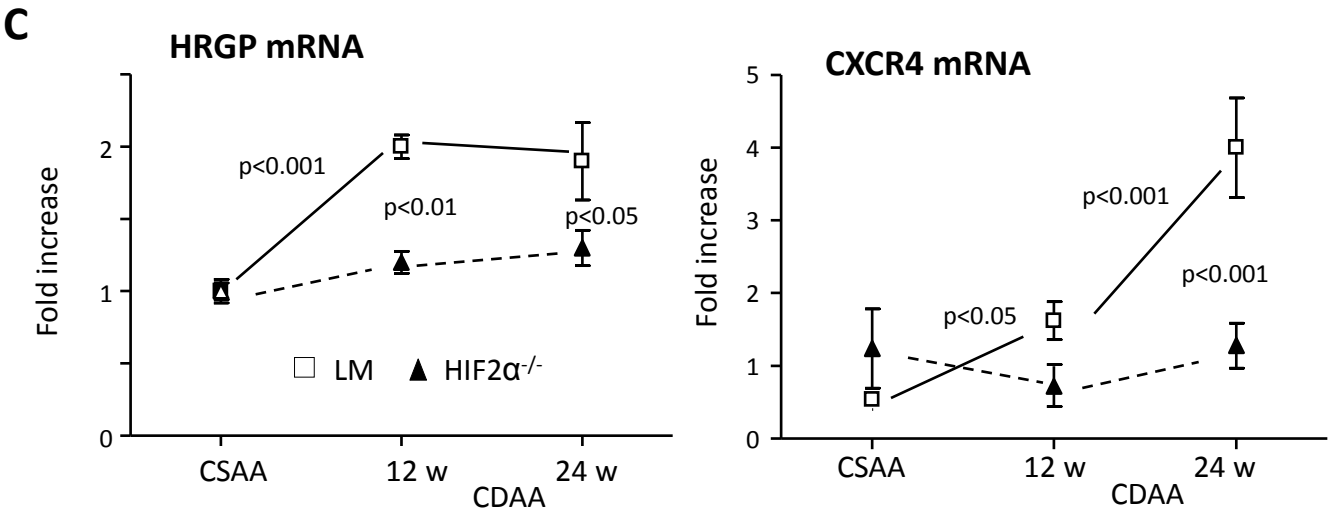

α-SMA immunohistochemistry
D
20 x
40 x
CSSA/CDAA diet
CSAA control mice (24 wk)
CSAA - HIF2α ko mice (24 wk)
CDAA control mice (12 wk)
CDAA - HIF2α ko mice (12 wk)
CDAA control mice (24 wk)
CDAA - HIF2α ko mice (24 wk)

Figure 5


A
Hypoxia
H/C
15'
30'
1h
3h
6h
16h
24h
48h
72h
HRGP
GAPDH


B
HRGP mRNA
p<0.01
2.0
1.5
0.2
0.1
0.0
2-ΔCT
HepG2 C
HepG2 EV
HepG2 HIF2α
HepG2 control
HepG2 empty vector
HepG2 overexpressing HIF2-α


C
HRGP mRNA
Fold increase
p<0.001
p<0.01
p<0.05
LM
HIF2α-/-
CSAA
12 w
24 w
CDAA
CXCR4 mRNA
Fold increase
p<0.001
p<0.05
p<0.001
CSAA
12 w
24 w
CDAA

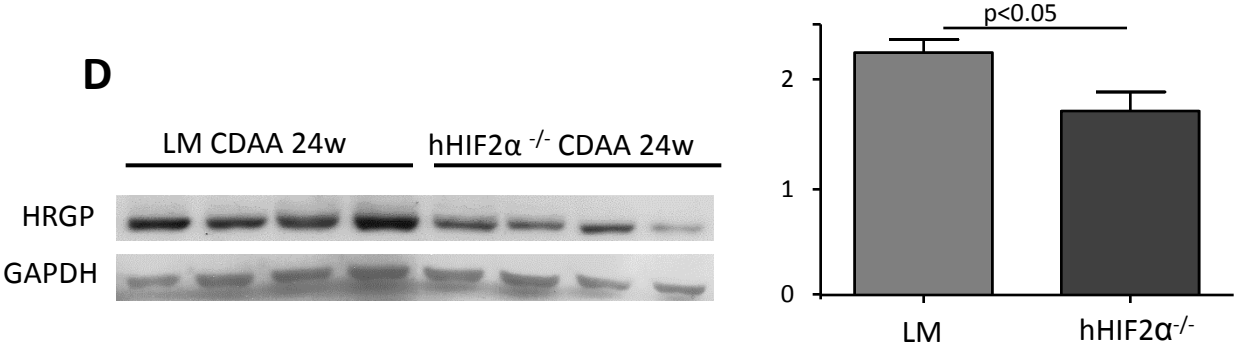
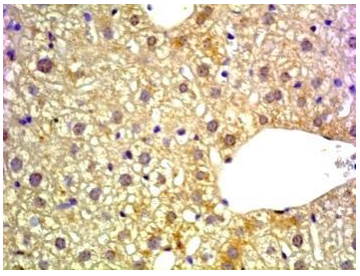

D
LM CDAA 24w
hHIF2α -/- CDAA 24w
HRGP
GAPDH
p<0.05
LM
hHIF2α-/-

Figure 5

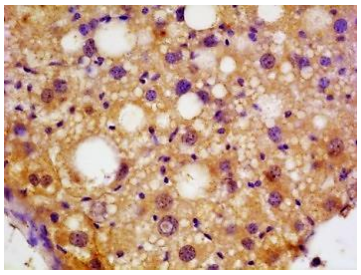
E

HRGP immunohistochemistry

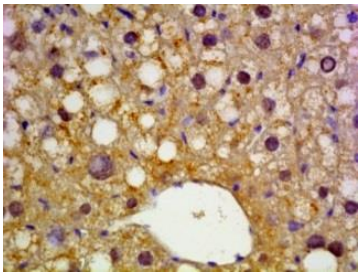
CSAA control mice (24 wk)



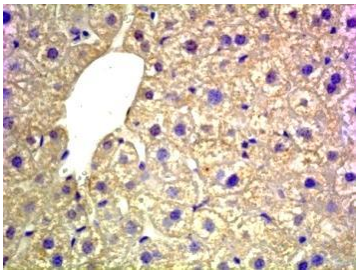
CDAA control mice (12 wk)



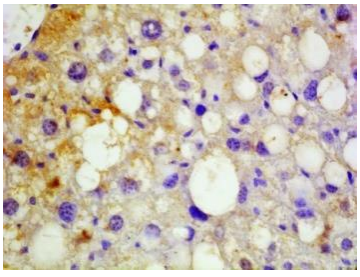
CDAA control mice (24 wk)



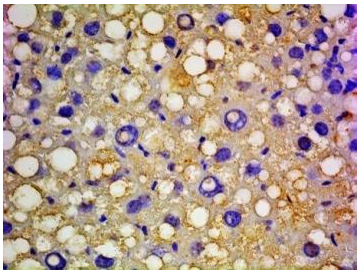
CSAA - HIF2α ko mice (24 wk)



CDAA - HIF2α ko mice (12 wk)



CDAA - HIF2α ko mice (24 wk)



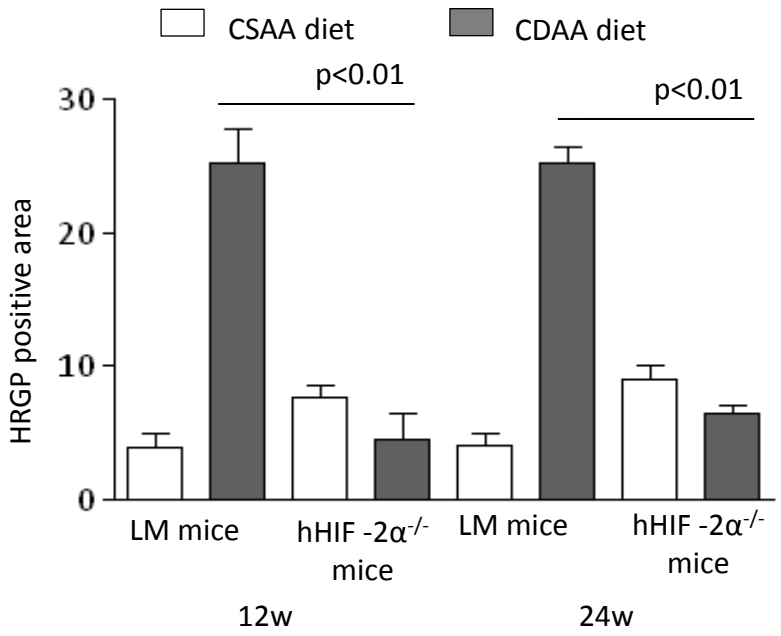
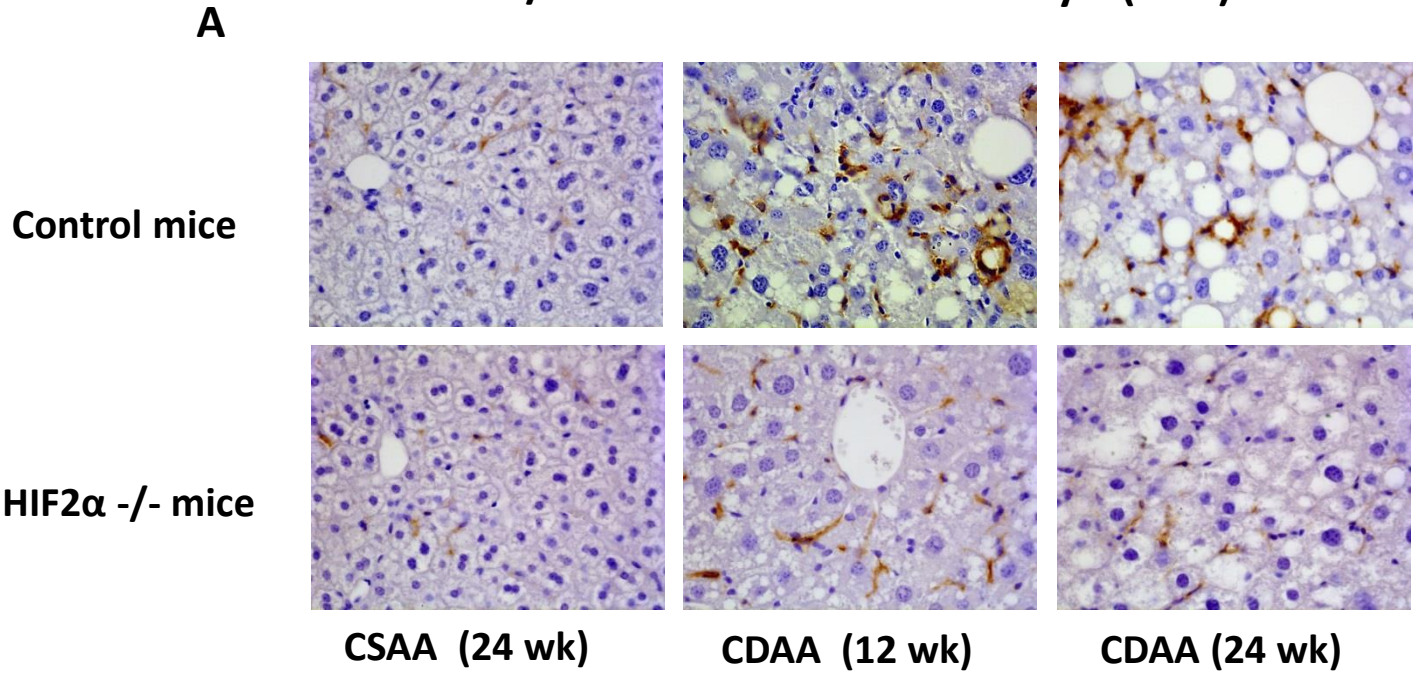


Figure 6

A


F4/80 immunohistochemistry (40 x)
Control mice
HIF2α -/- mice
CSAA (24 wk)
CDAA (12 wk)
CDAA (24 wk)

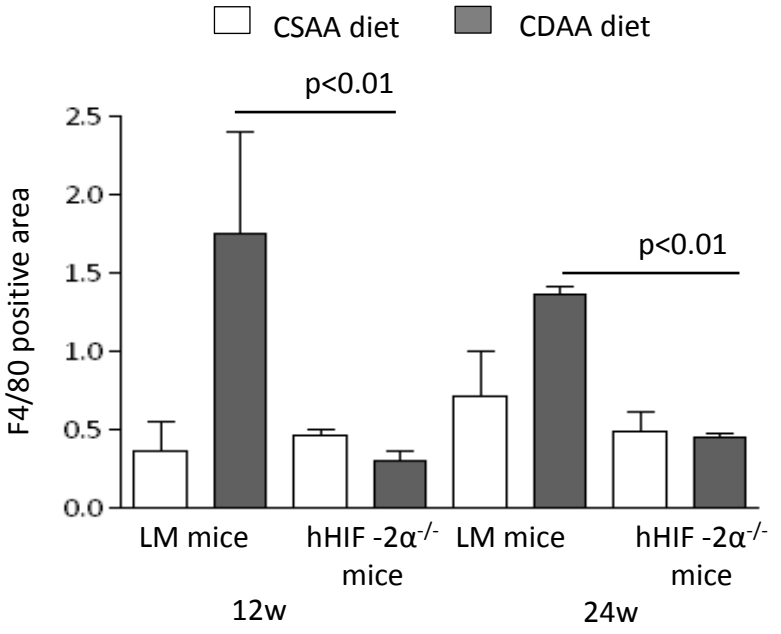
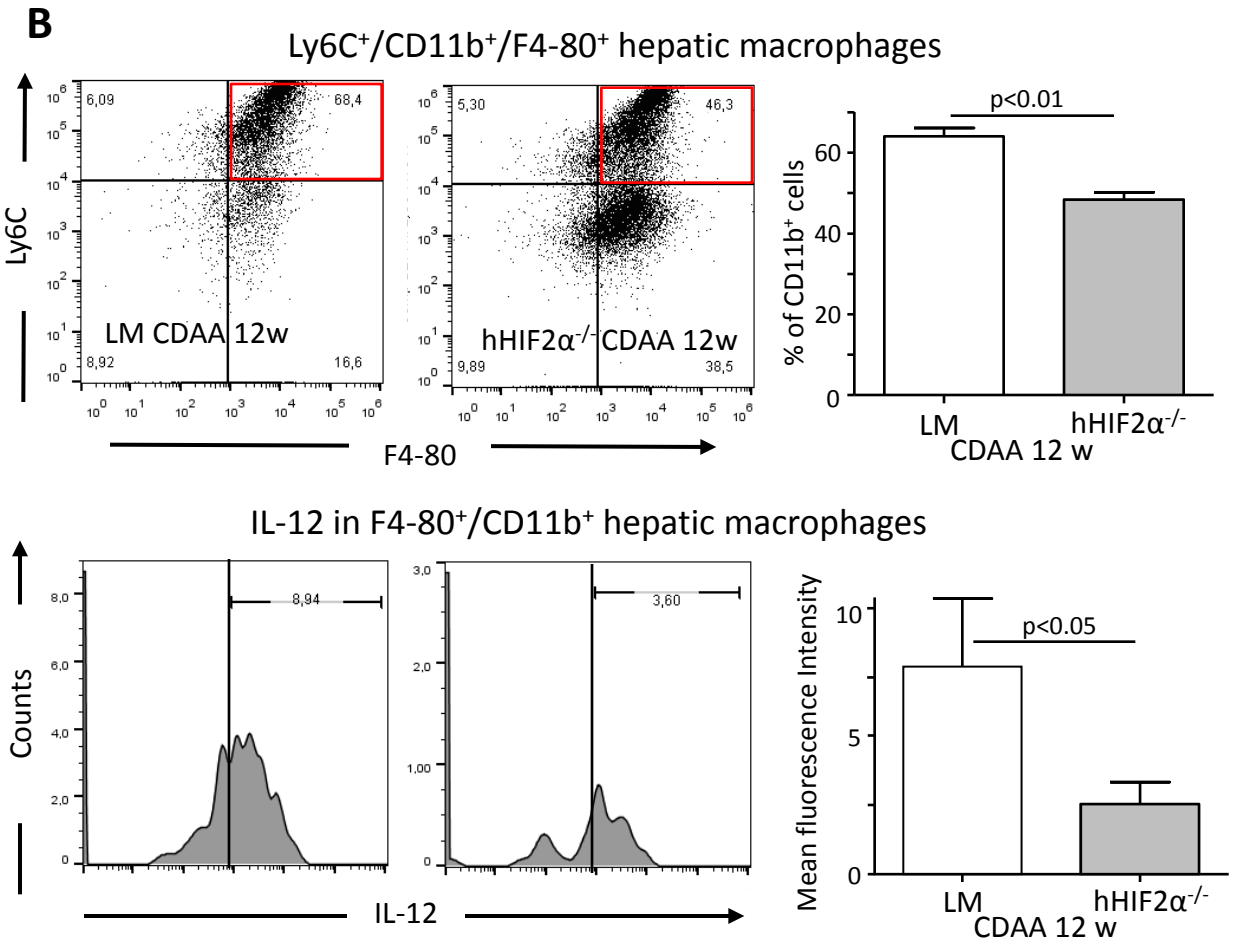

CSAA diet
CDAA diet
p<0.01
p<0.01
F4/80 positive area
2.5
2.0
1.5
1.0
0.5
0.0
LM mice
hHIF -2α-/- mice
LM mice
hHIF -2α-/- mice
12w
24w

Figure 6


B
Ly6C+/CD11b+/F4-80+ hepatic macrophages
Ly6C
F4-80
6,09
68,4
8,92
16,6
LM CDAA 12w
5,30
46,3
9,89
38,5
hHIF2α-/- CDAA 12w
p<0.01
% of CD11b+ cells
LM
hHIF2α-/-
CDAA 12 w
IL-12 in F4-80+/CD11b+ hepatic macrophages
Counts
IL-12
8,94
3,60
p<0.05
Mean fluorescence Intensity
LM
hHIF2α-/-
CDAA 12 w

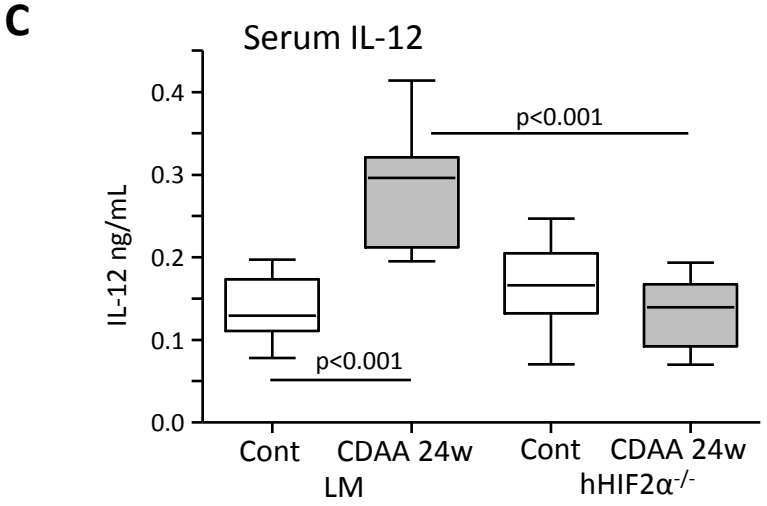

C
Serum IL-12
IL-12 ng/mL
p<0.001
p<0.001
Cont
CDAA 24w
Cont
CDAA 24w
LM
hHIF2α-/-

Figure 7

A

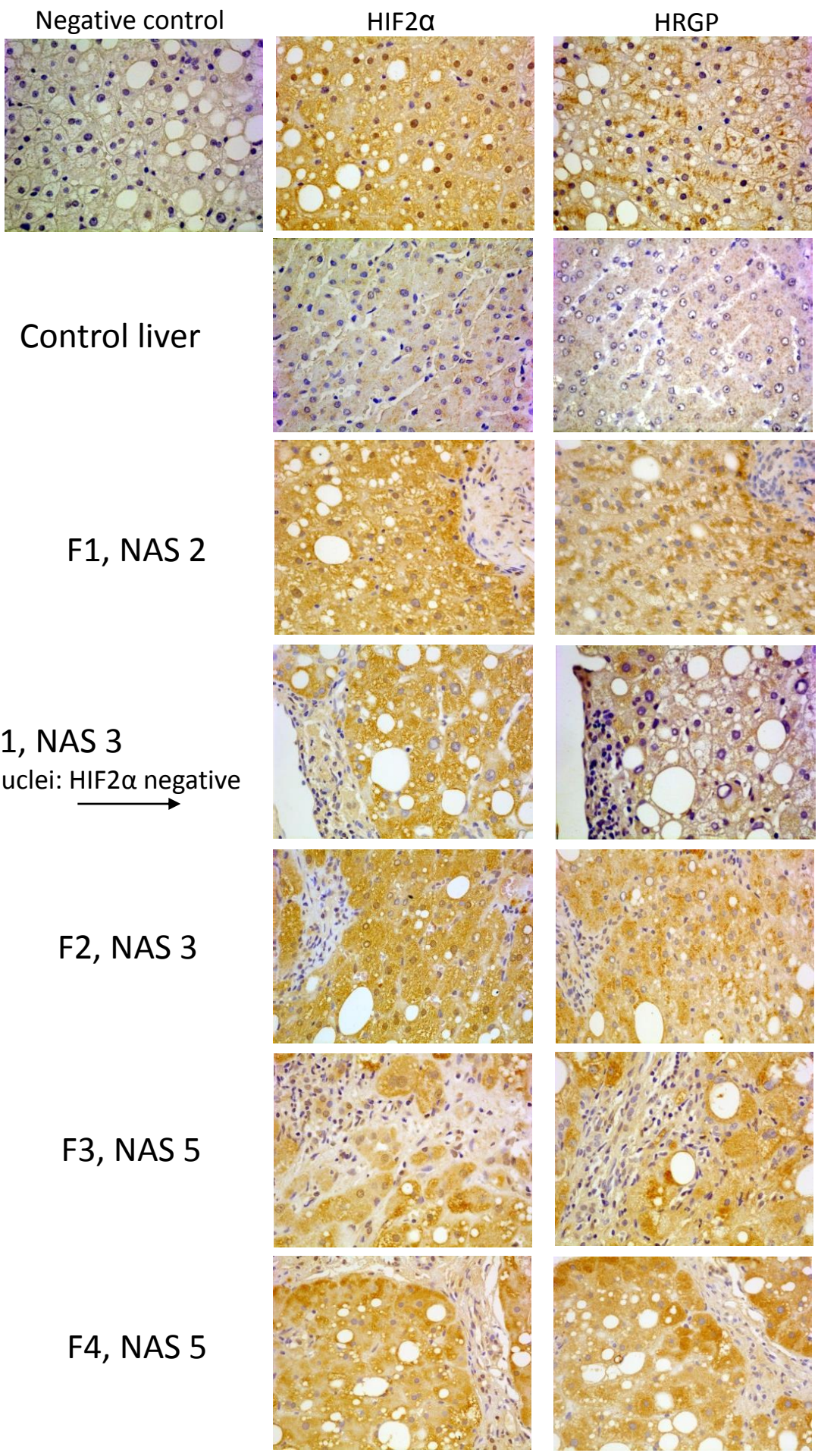
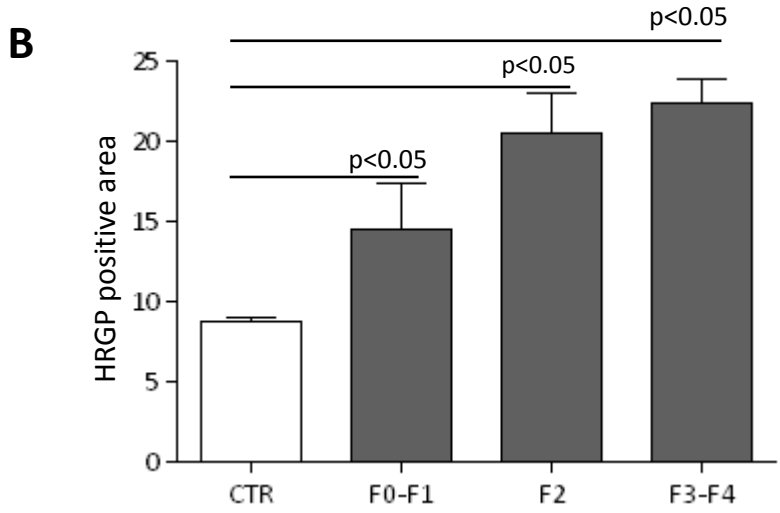
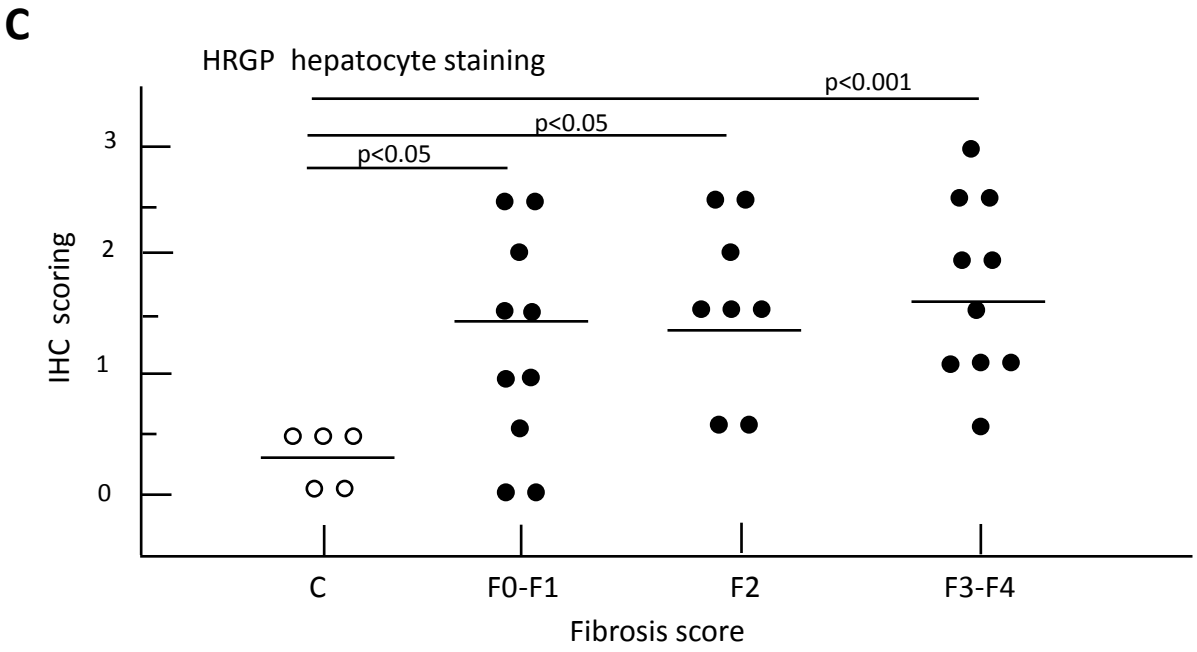
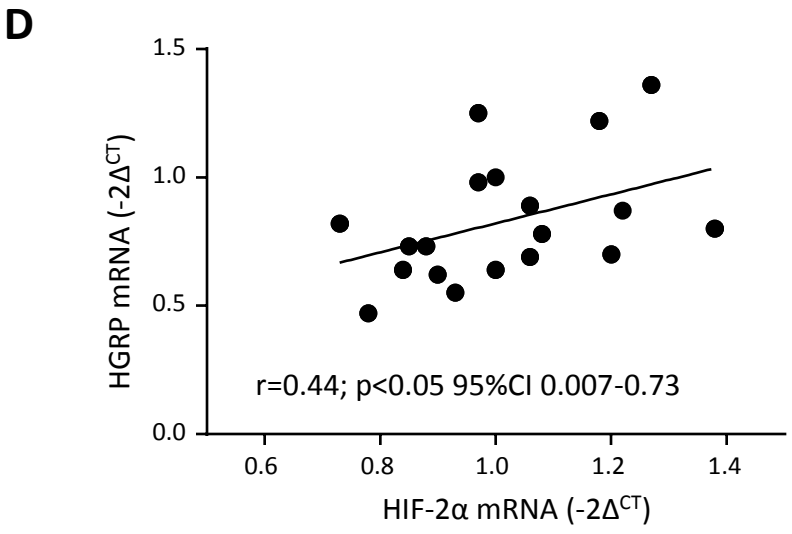

Negative control
HIF2α
HRGP
F0, NAS 4
Control liver
F1, NAS 2
F1, NAS 3
Nuclei: HIF2α negative
F2, NAS 3
F3, NAS 5
F4, NAS 5

Figure 7


B
p<0.05
p<0.05
p<0.05
HRGP positive area
25
20
15
10
5
0
CTR
F0-F1
F2
F3-F4


C
HRGP hepatocyte staining
p<0.001
p<0.05
p<0.05
IHC scoring
3
2
1
0
C
F0-F1
F2
F3-F4
Fibrosis score


D
HGRP mRNA (-2Δ^CT)
1.5
1.0
0.5
0.0
r=0.44; p<0.05 95%CI 0.007-0.73
0.6
0.8
1.0
1.2
1.4
HIF-2α mRNA (-2Δ^CT)

1.1 Supplementary information related to PAPER n.1

"Hypoxia-inducible factor 2α drives nonalcoholic fatty liver progression by triggering hepatocyte release of histidine rich glycoprotein."

Elisabetta Morello, Salvatore Sutti, Beatrice Foglia, Erica Novo, Stefania Cannito, Claudia Bocca, Martina Rajsky, Stefania Bruzzì, Maria Lorena Abate, Chiara Rosso, Cristina Bozzola, Ezio David, Elisabetta Bugianesi, Emanuele Albano, Maurizio Parola.

Hepatology. 2017 Dec 21. doi: 10.1002/hep.29754. Article in press.

Hypoxia-inducible factor 2α drives nonalcoholic fatty liver progression by triggering hepatocyte release of histidine rich glycoprotein

Elisabetta Morello[1]*, **Salvatore Sutti**[2,3]*, Beatrice Foglia[1], Stefania Cannito[1], Erica Novo[1], Claudia Bocca[1], Martina Rajsky[1], Stefania Bruzzì[3], Maria Lorena Abate[2], Chiara Rosso[2], Cristina Bozzola[3] Ezio David[4], Elisabetta Bugianesi[2], Emanuele Albano[3], Maurizio Parola[1§]

[1]Dept. Clinical and Biological Sciences, University of Torino, Italy.

[2]Dept. Medical Sciences, University of Torino, Italy.

[3]Dept. Health Sciences and Interdisciplinary Research Center for Autoimmune Diseases, University Amedeo Avogadro of East Piedmont, Novara, Italy.

[4]Pathology Unit, S. Giovanni Battista Hospital, Torino.

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Supplementary material and methods

Materials:

Enhanced chemiluminescence (ECL) reagents and nitrocellulose membranes (Hybond-C extra were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Polyclonal antibody against HIF-2α (NB100-122) was from Novus Biologicals (Cambridge, UK); GAPDH (sc-20357) was from Santa Cruz Biotechnology (Santa Cruz, CA, USA); mouse HRG (AF1905) was from R&D systems (Minneapolis, MN, USA) human HRG used for Western Blot detection (ab67807) was from Abcam (Cambridge, UK) and human HRG for immunohistochemistry experiments (HPA050269) was from Sigma (Sigma-Aldrich, St. Louis, MO, USA). Monoclonal antibodies for α-tubulin (T6074) and β-actin (A5441) were from Sigma (Sigma Aldrich Spa, Milan, Italy). Monoclonal antibodies against F4/80 for immunohistochemistry (14/4801/82) was from eBioscences (Affymetrix, St Clara, CA, USA) and α-SMA (M0851) was from DAKO (Agilent, St Clara, CA, USA). Lipofectamine 2000 (Invitrogen-Life Technologies), Plasmid DNA purification NucleoBond XtraMIDI (Macherey-Nagel, Germany), pCMV6-Entry vectors (Origene, Rockville, MD). RNA interference experiments to knockdown HIF-2α expression in HepG2 cells were performed using siRNA duplex (Qiagen Italia, Milano, Italy).

Immunohistochemistry, Sirius Red staining and histomorphometric analysis:

Paraffin-embedded human liver biopsies and/or murine liver specimens used in this study were immuno-stained as previously reported (1,2), and more details are reported in using polyclonal anti-HIF-2α antibody (dil. 1:100 v/v), polyclonal anti-HRGP antibody (dil. 1:50, v/v), monoclonal anti-α-SMA antibody (dil. 1:400, v/v) or F4-80 monocloclonal antibodies (dilution 1:1000, v/v). Collagen deposition was evidenced by Picro-Sirius Red staining as previously described, (30,31) and quantification of fibrosis in the murine liver was performed by histo-morphometric analysis using a digital camera and a bright field microscope to collect images that were then analyzed by employing the ImageJ software. (3) Hematoxilin/eosin stained mice liver sections were scored blind for steatosis and lobular inflammation. (4)

In vitro experiments and cell culture conditions:

In this study HepG2 cells (American Type Culture Collection, USA) were used and maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal-bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin and 25 μg/ml amphotericin-B, as previously reported. (1) In order to evaluate the HRGP protein levels under hypoxia condition, normal HepG2 cells were incubated in strictly controlled hypoxic conditions (3% O2) up to 72 hours.

The pCMV6-based mammalian expression vectors, empty (used as a control) and encoding HIF-2α (OriGene, Rockville, MD), were used in order to generate and select HepG2 cells stably overexpressing HIRF-2α. (28) HepG2 cells were seeded and then transfected 24 hr later with 10 μg of each vector using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). HIF-2α expression of the generated stable transfectants was carefully characterized, after which the cell lines carrying the

empty vector (EV) and overexpressing HIF-2α (HepG2 Hif-2α) were then used for the experiment described.

RNA interference experiments to knockdown HIF-2α expression in HepG2 cells were performed using siRNA duplex as previously described [1]. The following target sequence was used:

-HIF-2α: 5′-CCCGGATAGACTTATTGCCAA-3′.

The siRNA and related non-silencing controls were transfected in HepG2 cells with lipofectamine 2000 transfection reagent according to manufacturer's instructions up to 72 hrs. Transfected cells in fresh medium were then exposed for further 48 hrs to the desired experimental conditions and then harvested for sample preparation.

Western blot analysis:

Total cell lysates, obtained as previously described, (1,3) were subjected to sodium dodecyl sulfate-polyacrylamide gel-electrophoresis on 12%, 10% or 7.5% acrylamide gels, incubated with desired primary antibodies, then with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline-Tween containing 2% (w/v) non-fat dry milk and finally developed with the ECL reagents according to manufacturer’s instructions. Sample loading was evaluated by reblotting the same membrane with antibodies raised against GAPDH, α-tubulin and β-actin.

Supplementary Tables

Supplementary Table 1

Clinical and biochemical characterization of NAFLD patients investigated**.**

	Demographic Data
Patients Number (Male/Female)	27 (15/12)
Age (Years)	48 (23-70)
BMI	30.2 (22.3-39.6)
	Biochemical Data
HOMA-IR (n.v. <3)	3.7 (0.8-14.1)
AST (U/L– n.v. 5–40)	33.5 (16-93)
ALT (U/L n.v. 5–40)	44.1 (9-82)
γ-GT (U/L n.v. 5–45)	54.0 (11-525)
Fasting Glucose (mg/dL n.v. <100)	94.6 (72-197)
	Histological Data
Steatosis score	2 (0-3)
Inflammation score	1 (0-2)
Ballooning score	1 (0-2)
Fibrosis score	2 (0-4)
NAS score	4 (1-6)

The values are expressed as median and inter-quartile range (IQR). For histological scores the range of variability is included.

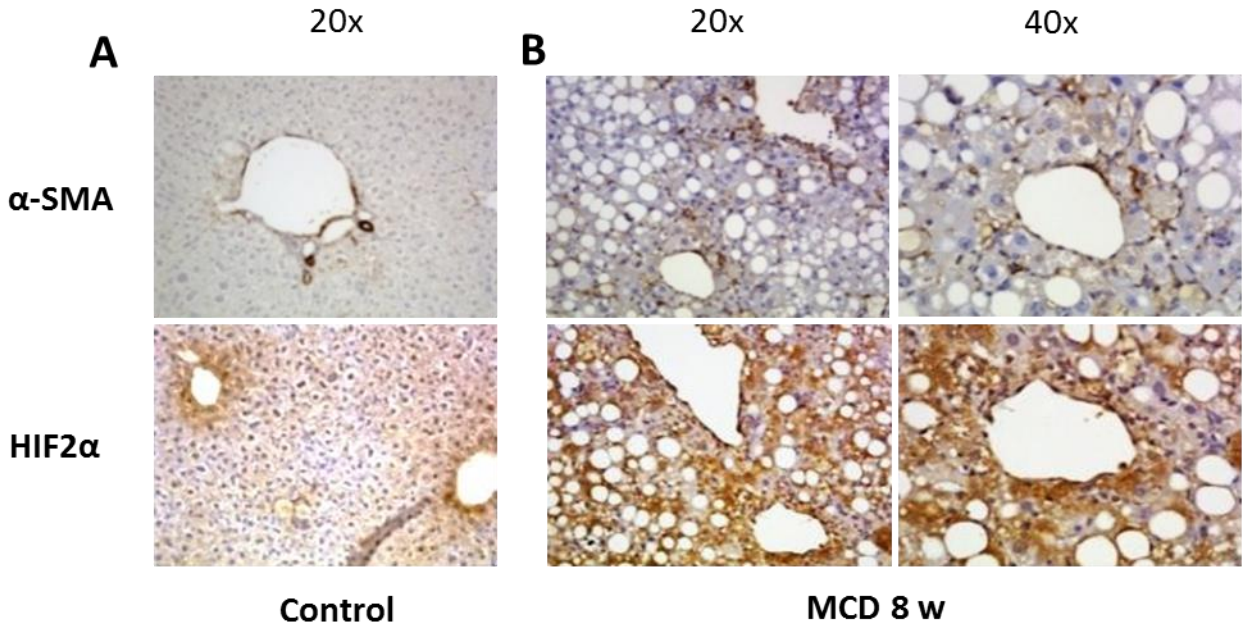
BMI, body mass index; AST, alanine aminotransferase; ALT, aspartate aminotransferase; γ-GT, gamma-glutamyl transpeptidase; HOMA-IR, homeostatic model assessment-insulin resistance; ISI, insulin sensitivity index; n.v., normal value

Supplementary Table 2

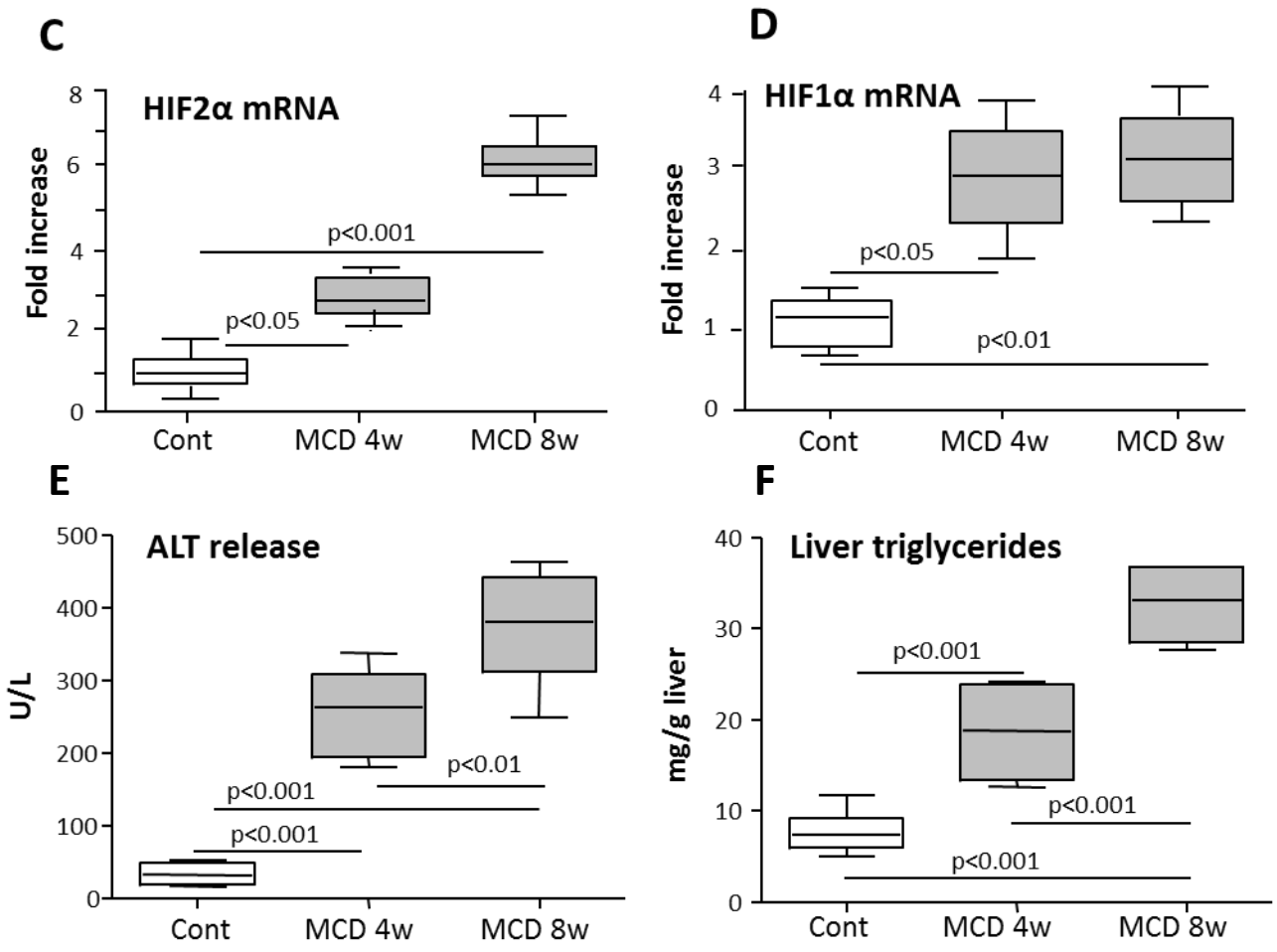
Oligonucleotide sequences of primers used for Q-PCR**.**

Gene	Primers
HIF1-α (mouse)	(FW) 5- TCAAGTTCAGCAACGTGGAAG -3 (RV) 5- TATCGAGGCTGTGTCGACTG -3
HIF2-α (mouse)	(FW) 5- CTAAGTGGCCTGTGGGTGAT -3 (RV) 5- GTGTCTTGGAAGGCTTGCTC-3
VEGF-A (mouse)	(FW) 5- CAGGCTGCTGTAACGATGAA-3 (RV) 5- TTTCTTGCGCTTTCGTTTTT -3
TGF-β (mouse)	(FW) 5- GGACTCTCCACCTGCAAGAC -3 (RV) 5- GACTGGCGAGCCTTAGTTTG -3
αSMA (mouse)	(FW) 5- CTGACAGAGGCACCACTGAA -3 (RV) 5- CATCTCCAGAGTCCAGCACA -3
COL1A1 (mouse)	(FW) 5- GAGCGGAGAGTACTGGATCG -3 (RV) 5- GTTCGGGCTGATGTACCAGT -3
TIMP1 (mouse)	(FW) 5- ATTCAAGGCTGTGGGAAATG -3 (RV) 5- CTCAGAGTACGCCAGGGAAC -3
β-ACTIN (mouse)	(FW) 5-AGCCATGTACGTAGCCATCC -3 (RV) 5- CTCTCAGCTGTGGTGGTGAA -3
HIF2-α (human)	(FW) 5- CGCTAGACTCCGAGAACAT -3 (RV) 5- GGCTTGAACAGGGATTCAGT -3
β-ACTIN (human)	(FW) 5- AGAGCTACGAGCTGCCTGAC -3 (RV) 5- GGATGCCACAGGACTGGA -3
HRG (human)	(FW) 5- GCCCGAAAAACCTTGTCATA -3 (RV) 5- CTAGATCCATGGGGCTTGAA -3
CXCR4 (mouse)	(FW) 5- TGGAACCGATCAGTGTGAGT -3 (RV) 5- TTGCCGACTATGCCAGTCAA -3
CXCR4 (human)	(FW) 5- TCCATTCCTTTGCCTCTTTTGC -3 (RV) 5- ACGGAAACAGGGTTCCTTCAT -3
FASN (mouse)	(FW) 5- TGGGTTCTAGCCAGCAGAGT -3 (RV) 5- ACCACCAGAGACCGTTATGC -3
SREBP1 (mouse)	(FW) 5- GATCAAAGAGGAGCCAGTGC -3 (RV) 5- TAGATGGTGGCTGCTGAGTG -3
EPO (human)	(FW) 5- GAGCCCAGAAGGAAGCCATC -3 (RV) 5- GCGGAAAGTGTCAGCAGTGA -3

Supplementary Figures

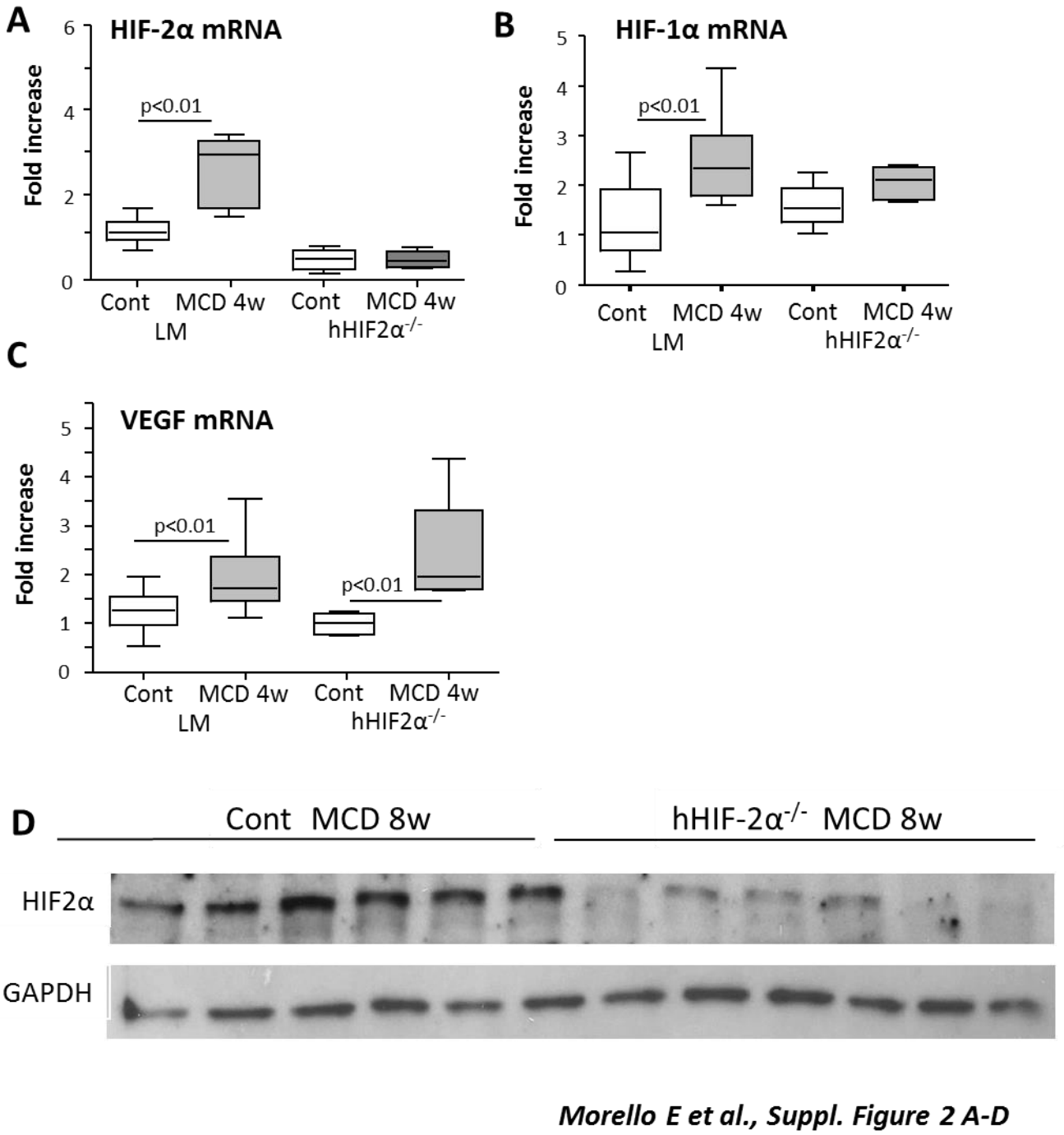


Morello E et al., Suppl. Figure 1A,B



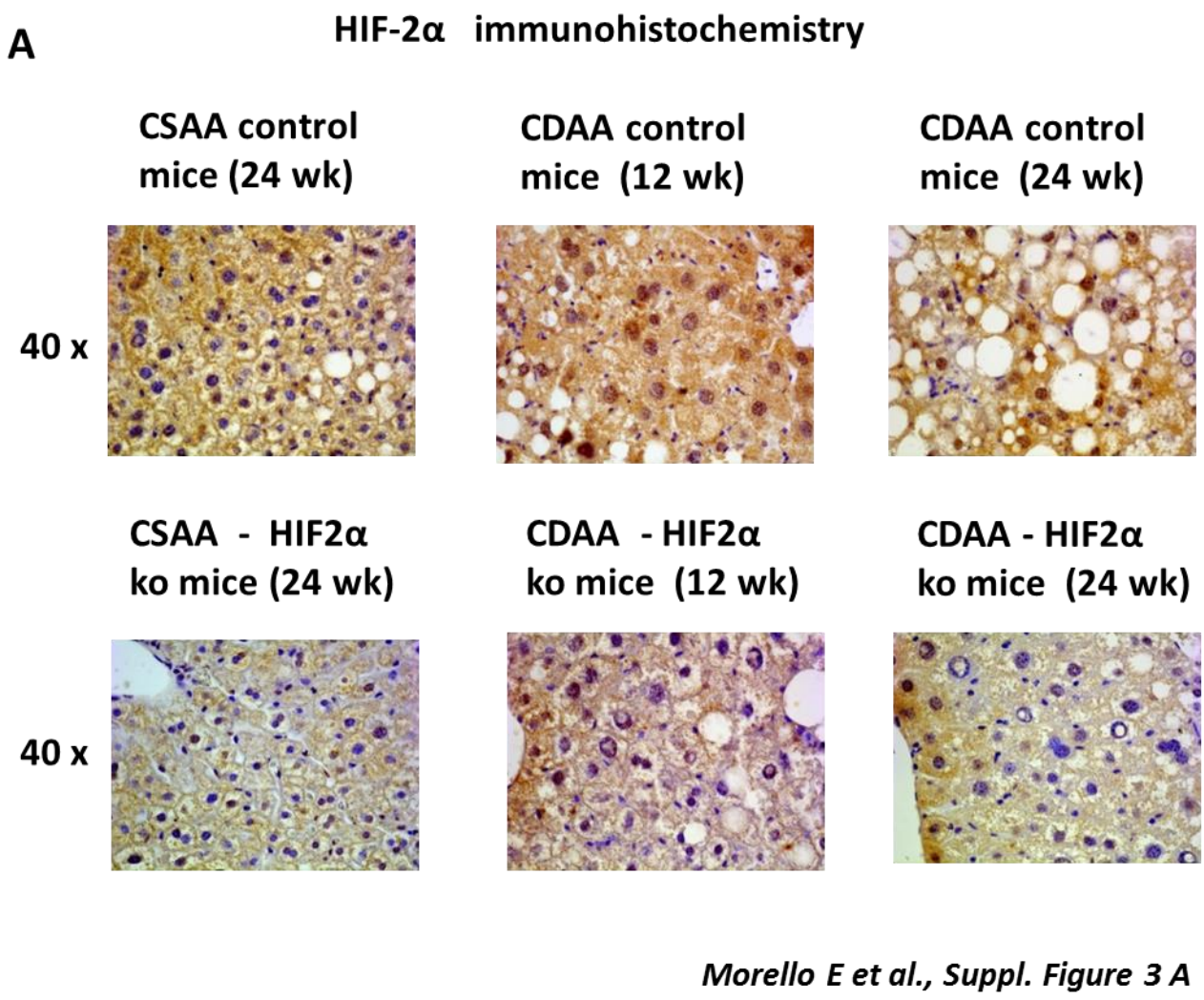
Morello et al. Suppl. Figure 1C-F

Suppl. Fig. 1. **Expression of HIF-2α in mice liver**. Immunohistochemistry analysis for HIF-2α and α-smooth muscle actin (α-SMA) in liver specimens from wild type mice fed with control (**A**) or MCD diet (8 weeks) (**B**). Original magnification as indicated. Liver expression of HIF-2α (**C**) and HIF-1α (**D**), evaluated by quantitative real-time PCR (Q-PCR) in MCD fed mice (4 and 8 weeks). Parenchymal injury and steatosis, estimated by measuring the circulating levels of alanine (ALT) **(E)** and hepatic TG content **(F)** are reported in MCD fed mice. The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression. The data are means ± SD of 5-7 animals per group. Boxes include the values within 25th and 75th percentile, whereas horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.



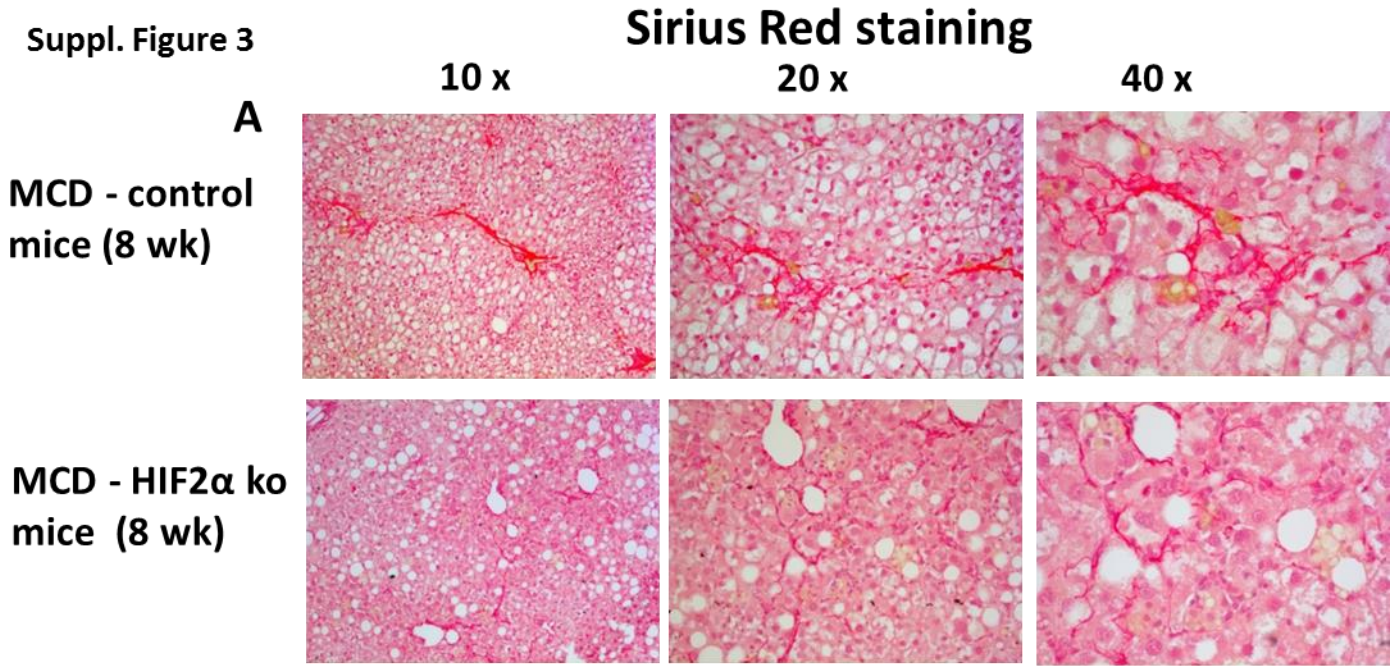
Morello E et al., Suppl. Figure 2 A-D

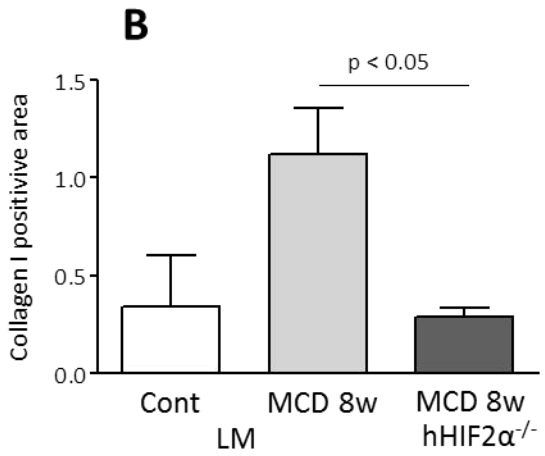
Suppl. Fig. 2. HIFs and VEGF gene expression in mice carrying hepatocyte-specific deletion of HIF-2α. Gene expression of HIF-2α (**A**), HIF-1α (**B**) and VEGF (**C**) evaluated by quantitative real-time PCR (Q-PCR) in livers of wild type littermates (LM) and hHIF2α$^{-/-}$ mice fed with the MCD diet for 4 weeks. The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression. The data are means ± SD of 5-7 animals per group. Boxes include the values within 25th and 75th percentile, whereas horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons. Western blot analysis of HIF-2α protein levels was performed on total liver extracts obtained from either LM and hHIF2α $^{-/-}$ mice fed MCD diet for 8 weeks (**D**).



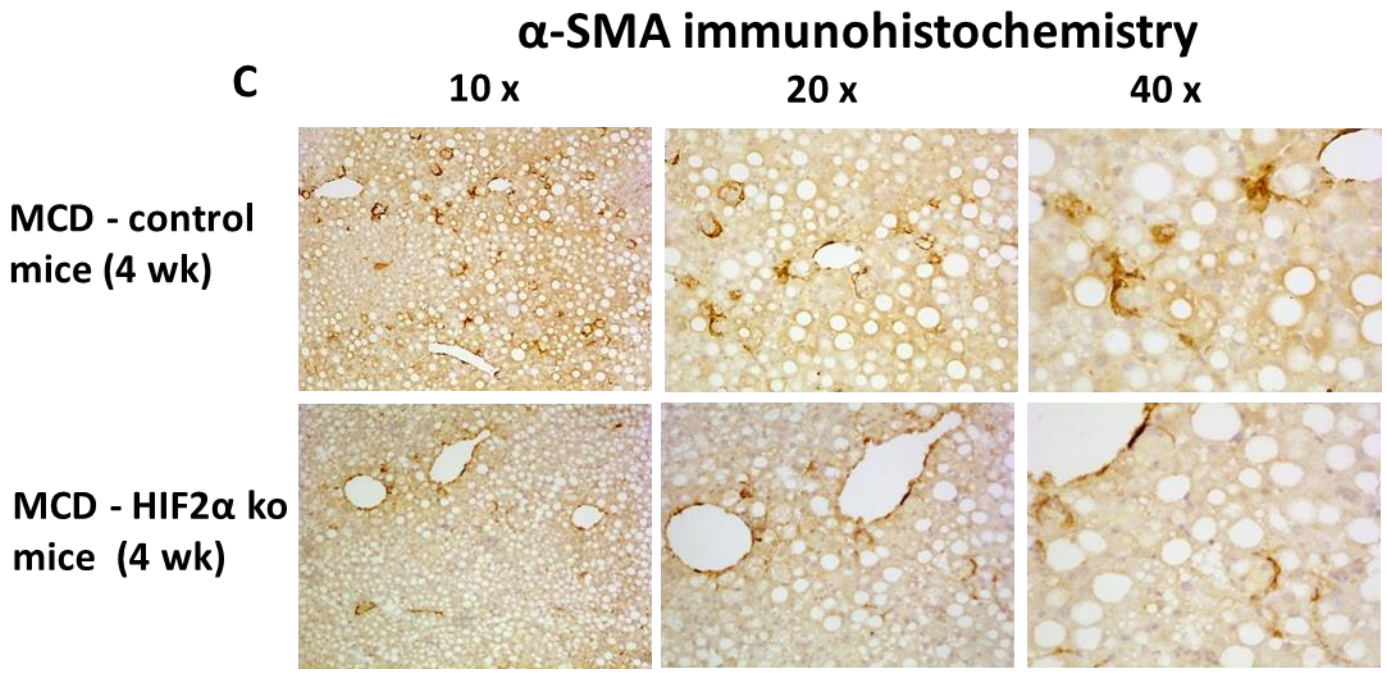
Suppl. Fig. 3. Expression of HIF-2α in mice liver. Immunohistochemistry analysis for HIF-2α in the livers of wild type littermates (LM) or hHIF-2α$^{-/-}$ CDAA or CSAA fed animals (12 and 24 weeks) (**A**). Original magnification as indicated.

Suppl. Figure 3

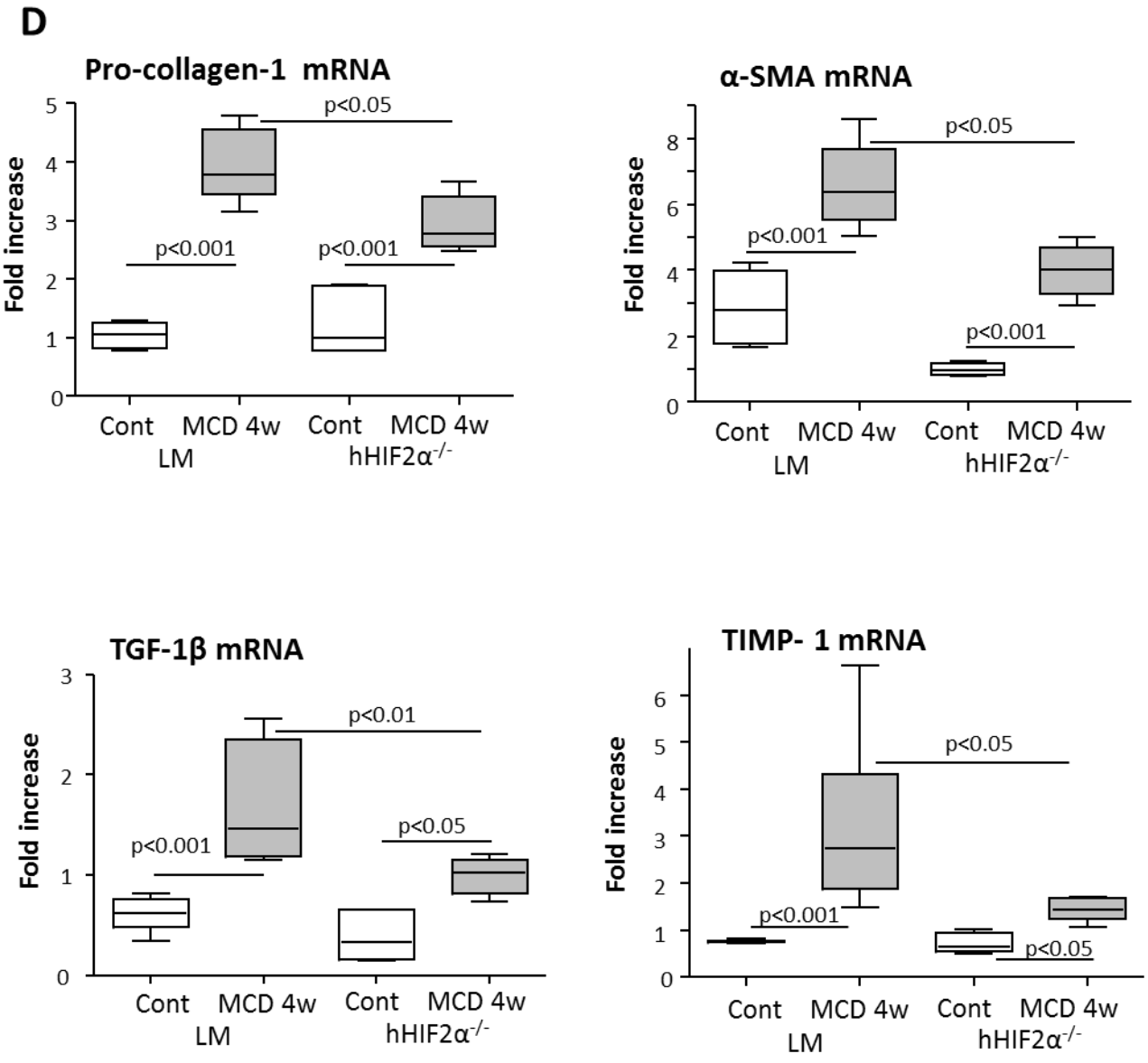




Morello E et al. Suppl. Figure 4 A,B

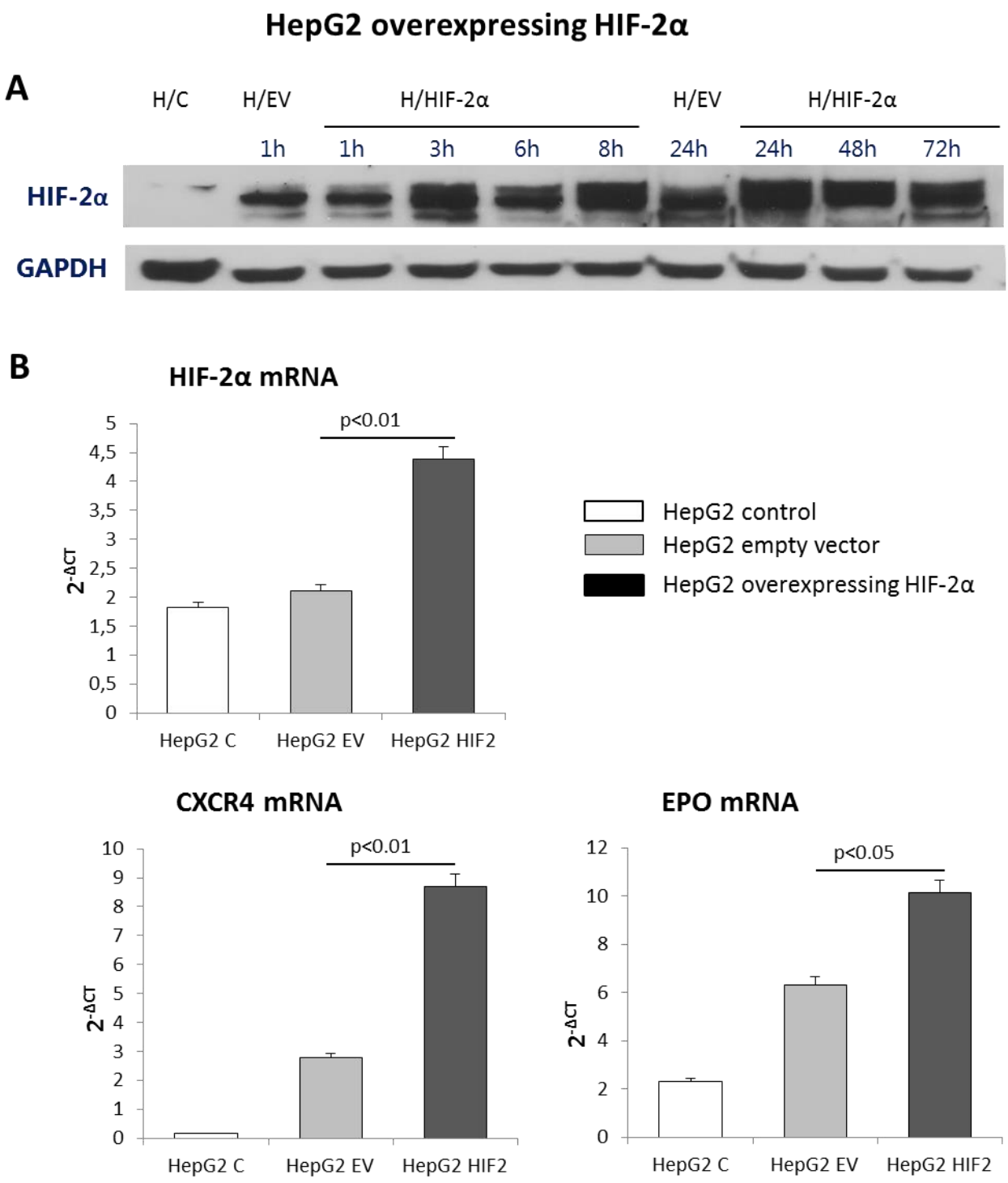


Morello E et al. Suppl. Figure 4 C

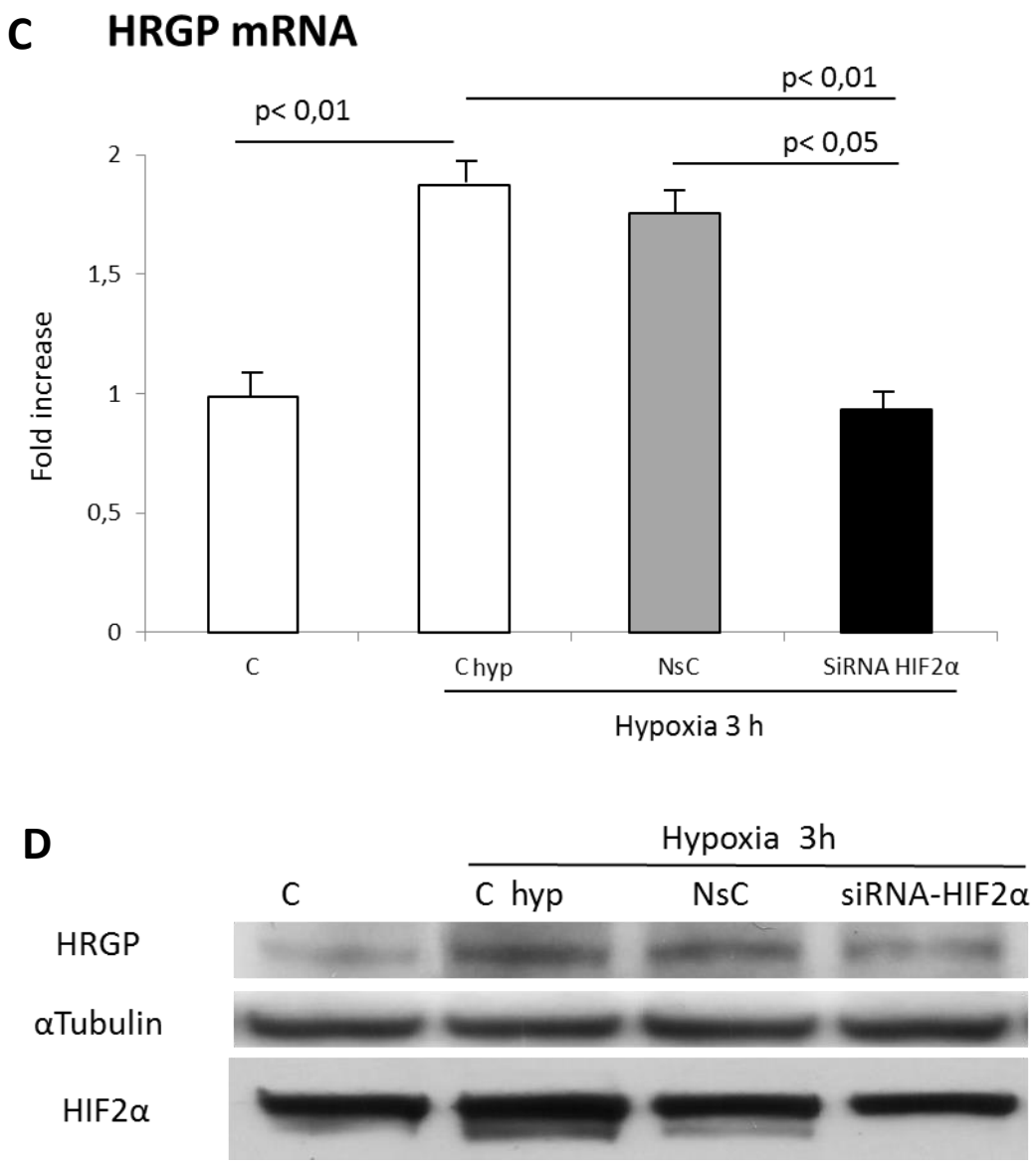


Morello E et al. Suppl. Figure 4 D

Suppl. Fig. 4 A-D. Effect on liver fibrosis of hepatocyte deletion of HIF-2α. hHIF-2$\alpha^{-/-}$ mice and wild type littermates (LM) were fed with MCD diet for 8 weeks. Liver fibrosis was morphologically evaluated by Sirius red staining (**A**). Original magnification as indicated. ImageJ software analysis was performed for Sirius red staining (**B**) to evaluate the amount of fibrosis. Immunohistochemistry for α-SMA (**C**) in the same animals. Data in graphs are expressed as means ± SEM. Analysis by quantitative real-time PCR (Q-PCR) of transcript levels of pro-fibrogenic genes pro-collagen 1A1, αSMA, TGFβ1 and TIMP1 in the different experimental groups (**D**). The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression. The data are means ± SD of 6-7 animals per group. Boxes include the values within 25th and 75th percentile, whereas horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise 80% of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

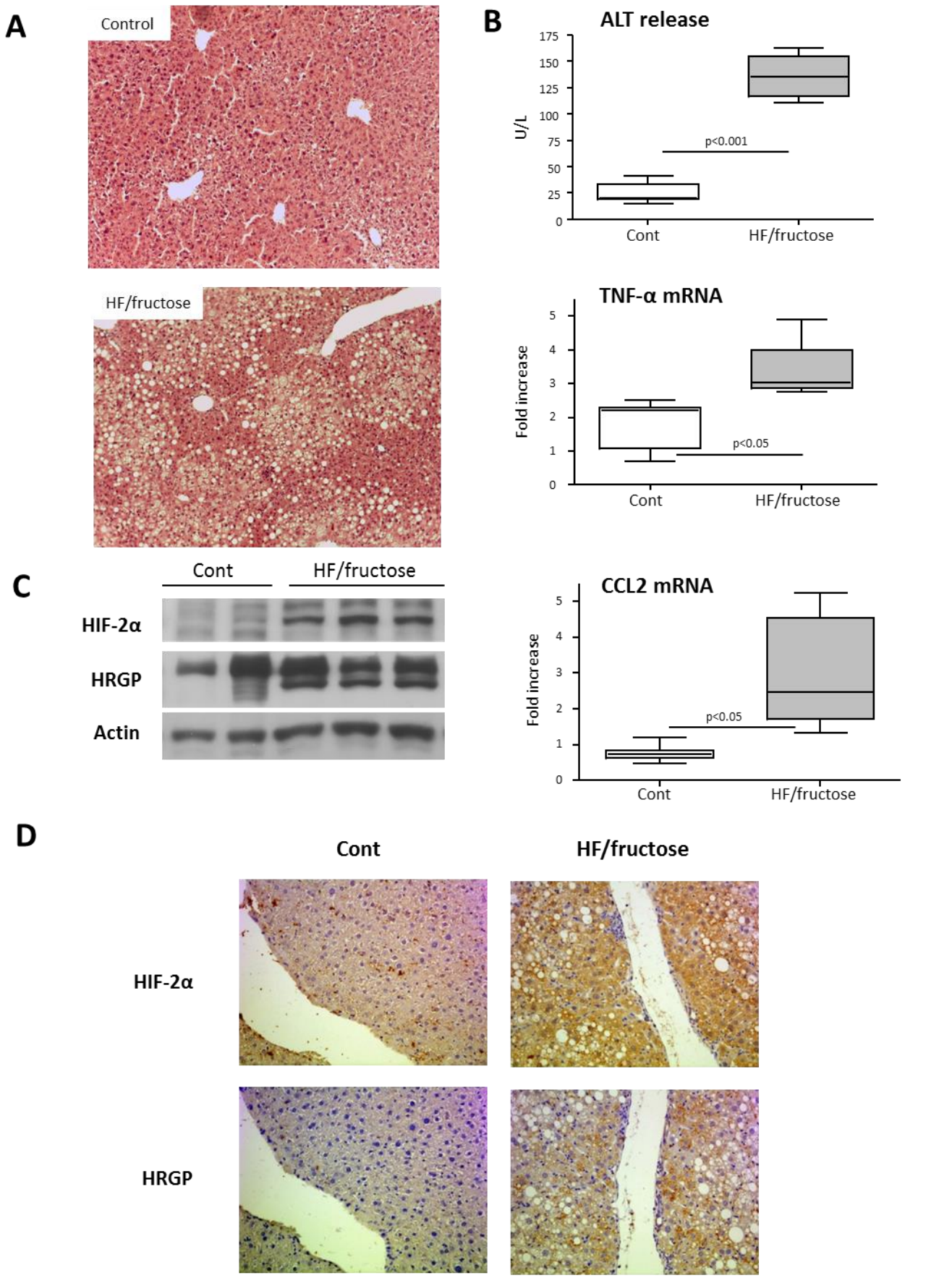


Morello E et al., Suppl. Figure 5 A-B



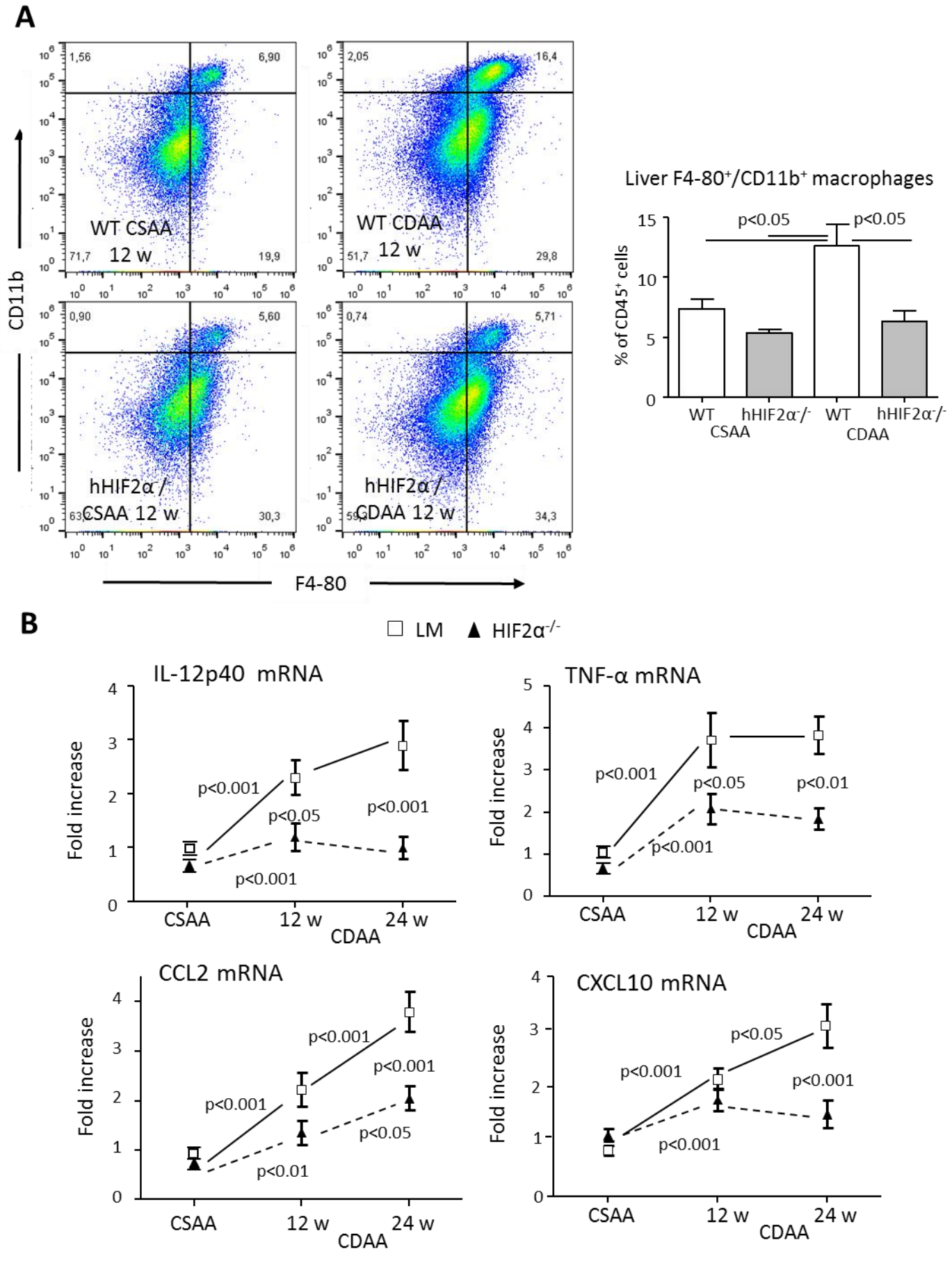
Morello E et al., Suppl. Figure 5 C

Suppl. Fig. 5. Direct relationships between HIF2α and HRGP expression. Western blotting analysis of HIF-2α levels in control HepG2 cells or HepG2 cells stably transfected to over-express HIF-2α exposed to hypoxic conditions (H/C: not transfected; H/EV: transfected with empty vector; H/HIF-2α: overexpressing HIF-2α) (**A**). Q-PCR analysis of HIF-2α and related targets transcript, including CXCR4 and erythropoietin (EPO) in control HepG2 cells (HepG2 C: not transfected; EV: transfected with empty vector) or HepG2 cells stably transfected to over-express HIF-2α (**B**). Q-PCR **(C)** and WB analysis **(D)** of HRGP transcript and protein levels in untreated HepG2 in normoxic conditions (C) or in HepG2 exposed for three hours to hypoxic conditions only (C hyp) as previously described (1) or in HepG2 exposed to hypoxia following silencing for HIF-2α with a specific siRNA (siRNA-HIF2α) or treatment with a non-silencing siRNA (NsC) **(C,D)**. WB analysis is offered also for HIF2α protein levels **(D)**. Data in graphs are expressed as means ± SEM. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.



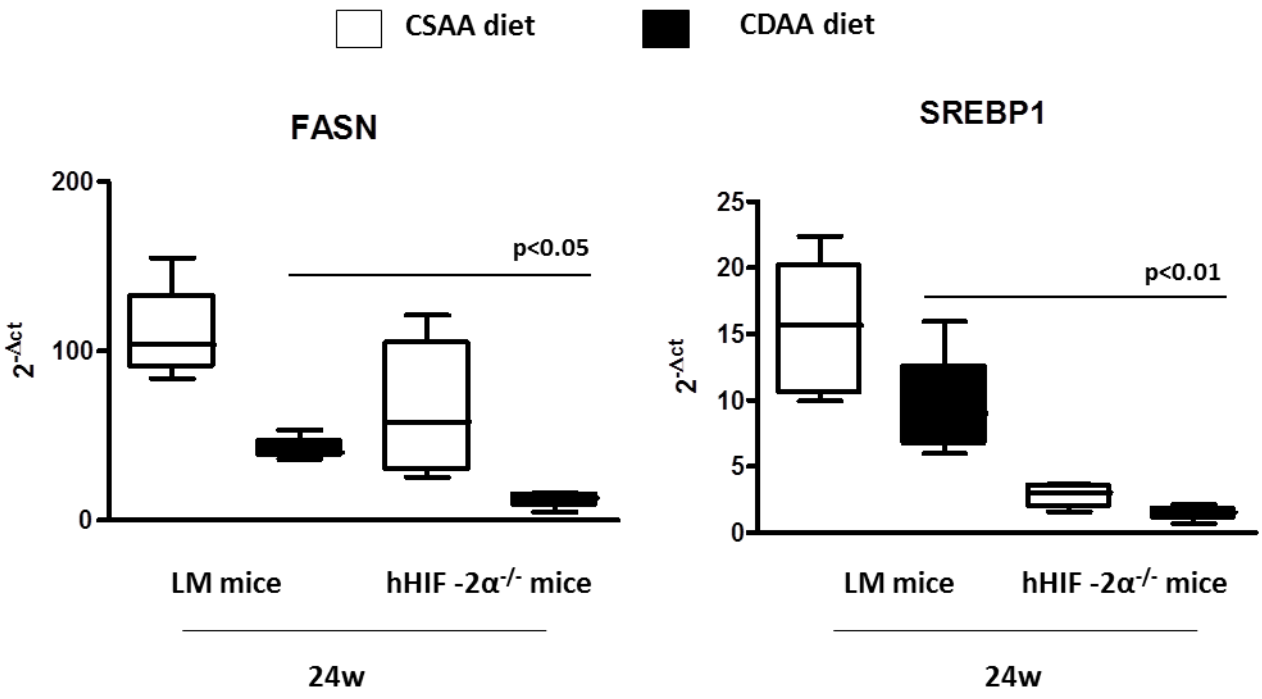
Morello E et al., Suppl. Figure 6 A-D

Suppl. Fig. 6. HIF-2α and HRGP expression in mice fed on High Fat / Fructose diet. C57BL/6 wild type mice were fed for 24 weeks with an high fat diet (58% of energy derived from fat, 18% from protein, and 24% from carbohydrates; 5.6 kcal/g) (Envigo RMS, S Pietro al Natisone, Italy) supplemented with 15% fructose (wt/v) in the drinking water. Liver morphology in mice exposed to HF/fructose mice diet or related control diet was evaluated by hematoxylin/eosin staining (magnification 10X) (**A**). Parenchymal injury was evaluated by measuring the serum levels of alanine (ALT) and pro-inflammatory citokynes mRNA levels (**B**). Elevation of both HIF-2α and HRGP protein levels was detected by Western blotting (**C**) and immune-histochemistry (IHC) analyses (magnification 20X) (**D**). The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression and are means ± SD of 6-8 animals per group. The boxes include the values within 25^{th} and 75^{th} percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10^{th}-90^{th} percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

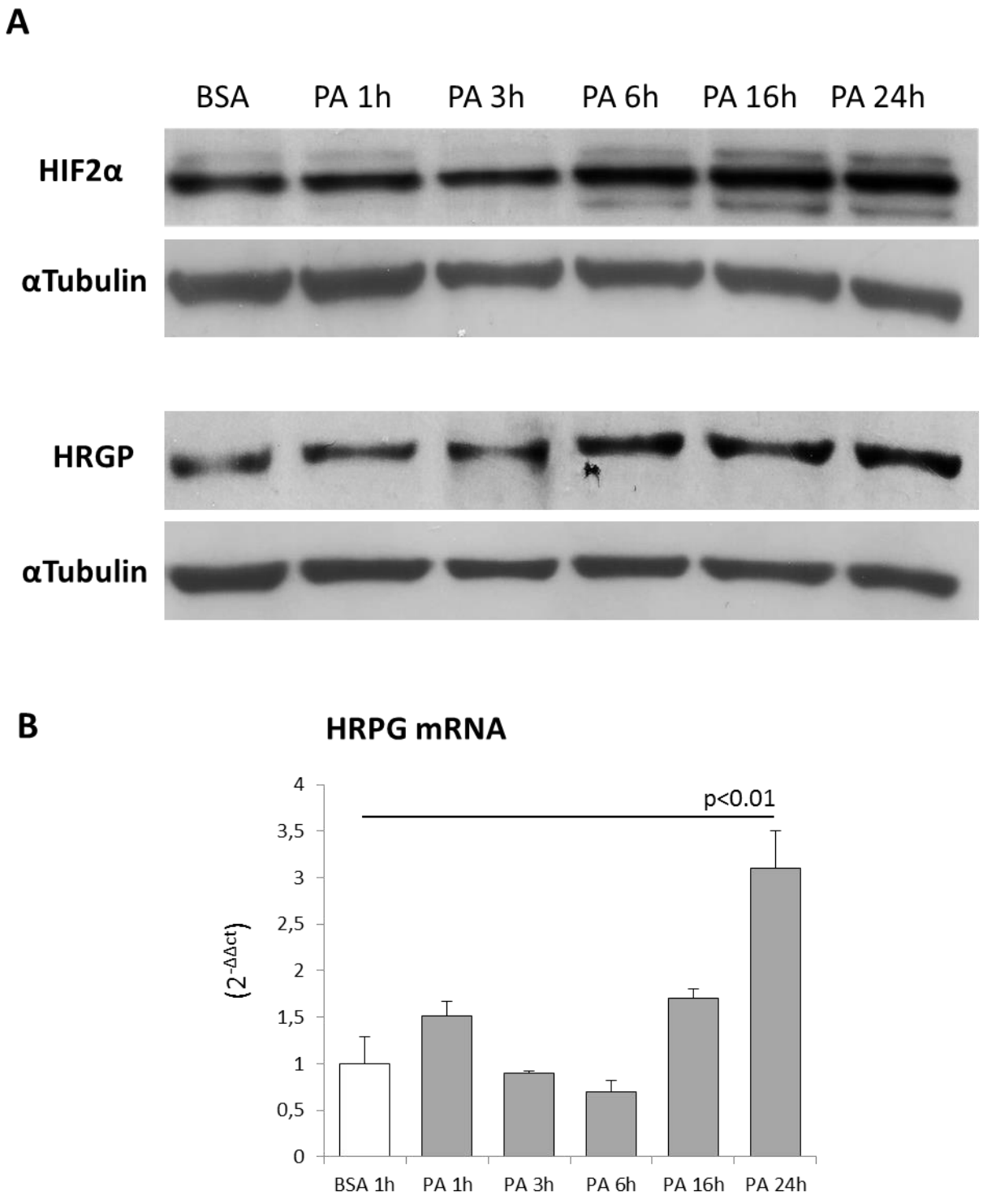


Morello E et al., Suppl. Figure 7 A-B

Suppl. Fig. 7. HIF2α-deficiency regulates macrophage infiltration. Hepatic myeloid cells were isolated from livers of either hHIF-2α$^{-/-}$ mice and wild type littermates (LM) fed with the CDAA diet analyzed by flow cytometry for Cd11b expression in CD45/F4-80-positive liver macrophages (**A**). Liver expression of inflammatory markers IL-12p40, TNF-α, CCL2 and CXCL10 was evaluated by quantitative real-time PCR (Q-PCR). The mRNA values are expressed as fold increase over control values after normalization to the β-actin gene expression and are means ± SD of 6-8 animals per group (**B**). Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.



Suppl. Fig. 8. HIF-2α modulates lipid homeostatic genes in the liver. qPCR analysis of sterol regulatory element binding factor -1 (SREBP1) and fatty acid synthase (FASN) in livers from hHIF-2α-/- mice vs related control littermates exposed to CDAA or CSAA diet. The mRNA values are expressed after normalization to the β-actin gene expression and are means ± SD of 6-8 animals per group. The boxes include the values within 25th and 75th percentile, while the horizontal bars represent the medians. The extremities of the vertical bars (10th-90th percentile) comprise the eighty percent of the values. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.



Morello E et al., Suppl. Figure 9 A - B

Suppl. Fig. 9. Murine hepatocytes treated with palmitic acid. Western blotting analysis of both HIF-2α and HRGP levels in AML12 cell line treated with palmitic acid 0,25mM up to 24hrs (**A**). Q-PCR analysis of HRGP in AML12 hepatocytes treated with palmitic acid 0,25mM up to 24hrs (**B**). Data in graphs are expressed as means ± SEM. Statistical differences were assessed by one-way ANOVA test with Tukey's correction for multiple comparisons.

References for Supplementary Material

1. Cannito S, Turato C, Paternostro C, Biasiolo A, Colombatto S, Cambieri I, et al. Hypoxia up-regulates SERPINB3 through HIF-2α in human liver cancer cells. Oncotarget 2015; 6:2206-2221.

2. Novo, E, Busletta C, Valfrè di Bonzo L, Povero D, Paternostro C, Mareschi K, et al. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. J Hepatol 2011;54:964-974.

3. Novo E, Villano G, Turato C, Cannito S, Paternostro C, Busletta C, et al. SerpinB3 promotes pro-fibrogenic responses in activated hepatic stellate cells. Scientific Reports 2017;7(1):3420.

4. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology 2005;41:1313-1321.

2. PAPER n.2

"SerpinB3 Promotes Pro-fibrogenic Responses in Activated Hepatic Stellate Cells."

Erica Novo, Gianmarco Villano, Cristian Turato, Stefania Cannito, Claudia Paternostro, Chiara Busletta, Alessandra Biasiolo, Santina Quarta, Elisabetta Morello, Claudia Bocca, Antonella Miglietta, Ezio David, Salvatore Sutti, Mario Plebani, Emanuele Albano, Maurizio Parola & Patrizia Pontisso.

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SCIENTIFIC REPORTS

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SerpinB3 Promotes Pro-fibrogenic Responses in Activated Hepatic Stellate Cells

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Erica Novo[1], Gianmarco Villano[2], Cristian Turato[3], Stefania Cannito[1], Claudia Paternostro[1], Chiara Busletta[1], Alessandra Biasiolo[2], Santina Quarta[2], Elisabetta Morello[1], Claudia Bocca[1], Antonella Miglietta[1], Ezio David[4], Salvatore Sutti[5], Mario Plebani[2], Emanuele Albano[5], Maurizio Parola[1] & Patrizia Pontisso[2]

SerpinB3 is a hypoxia- and hypoxia-inducible factor-2α-dependent cystein protease inhibitor that is up-regulated in hepatocellular carcinoma and in parenchymal cells during chronic liver diseases (CLD). SerpinB3 up-regulation in CLD patients has been reported to correlate with the extent of liver fibrosis and the production of transforming growth factor-β1, but the actual role of SerpinB3 in hepatic fibrogenesis is still poorly characterized. In the present study we analyzed the pro-fibrogenic action of SerpinB3 in cell cultures and in two different murine models of liver fibrosis. "*In vitro*" experiments revealed that SerpinB3 addition to either primary cultures of human activated myofibroblast-like hepatic stellate cells (HSC/MFs) or human stellate cell line (LX2 cells) strongly up-regulated the expression of genes involved in fibrogenesis and promoted oriented migration, but not cell proliferation. Chronic liver injury by CCl_4 administration or by feeding a methionine/choline deficient diet to transgenic mice over-expressing human SerpinB3 in hepatocytes confirmed that SerpinB3 over-expression significantly increased the mRNA levels of pro-fibrogenic genes, collagen deposition and αSMA-positive HSC/MFs as compared to wild-type mice, without affecting parenchymal damage. The present study provides for the first time evidence that hepatocyte release of SerpinB3 during CLD can contribute to liver fibrogenesis by acting on HSC/MFs.

Liver fibrogenesis is a dynamic and highly integrated process that, irrespective of the etiology, results in a progressive accumulation of extracellular matrix (ECM) components and drives the progression of chronic liver disease (CLD)[1–3]. A major pro-fibrogenic role is played by hepatic myofibroblasts (MFs)[1–9] which mainly originate from a process of activation/trans-differentiation of hepatic stellate cells (HSC) or HSC/MFs[8,9]. The persistent activation of these cells is the consequence of a complex interaction between growth factors, cytokines, chemokines, reactive oxygen species (ROS) and other mediators[1–7]. In the pro-fibrogenic environment these factors are released by- and interact with- several liver cell populations, including damaged hepatocytes, activated inflammatory cells and MFs[1–7]. Accordingly, the identification and characterization of the mediators involved in sustaining the pro-fibrogenic role of MFs during CLD progression is a critical issue to design novel selective therapeutic strategies and/or to develop diagnostic procedures.

Along these lines, SerpinB3, a member of the family of serine-proteases inhibitors (serpins)[10,11], has recently emerged as a mediator involved in CLD progression[12,13] and in liver carcinogenesis[14–18]. SerpinB3 expression is regulated by hypoxia through the hypoxia-inducible factor-2α (HIF-2α)[19]. In normal human and murine livers SerpinB3 is virtually undetectable, but its expression is readily appreciable in a significant percentage of liver biopsies from CLD patients, as well as in hepatocellular carcinoma (HCC)[12–18] and hepatoblastomas[20]. In both neoplastic and non-neoplastic settings, liver SerpinB3 up-regulation correlates with that of transforming growth factor-β1 (TGF-β1), a critical mediator in liver fibrogenesis[12,21]. In particular, a study performed in liver biopsies from 94 patients with CLD of different etiology has outlined a significant "*in vivo*" correlation between TGF-β1

[1]Department Clinical and Biological Sciences, Unit of Experimental Medicine and Clinical Pathology, University of Torino, Torino, Italy. [2]Department of Medicine, University of Padova, Padova, Italy. [3]Veneto Institute of Oncology IOV – IRCCS, Padua, Italy. [4]Pathology Unit, S. Giovanni Battista Hospital, Torino, Italy. [5]Department of Health Sciences, University "A. Avogadro" of East Piedmont, Novara, Italy. Erica Novo and Gianmarco Villano contributed equally to this work. Correspondence and requests for materials should be addressed to M.P. (email: maurizio.parola@unito.it)

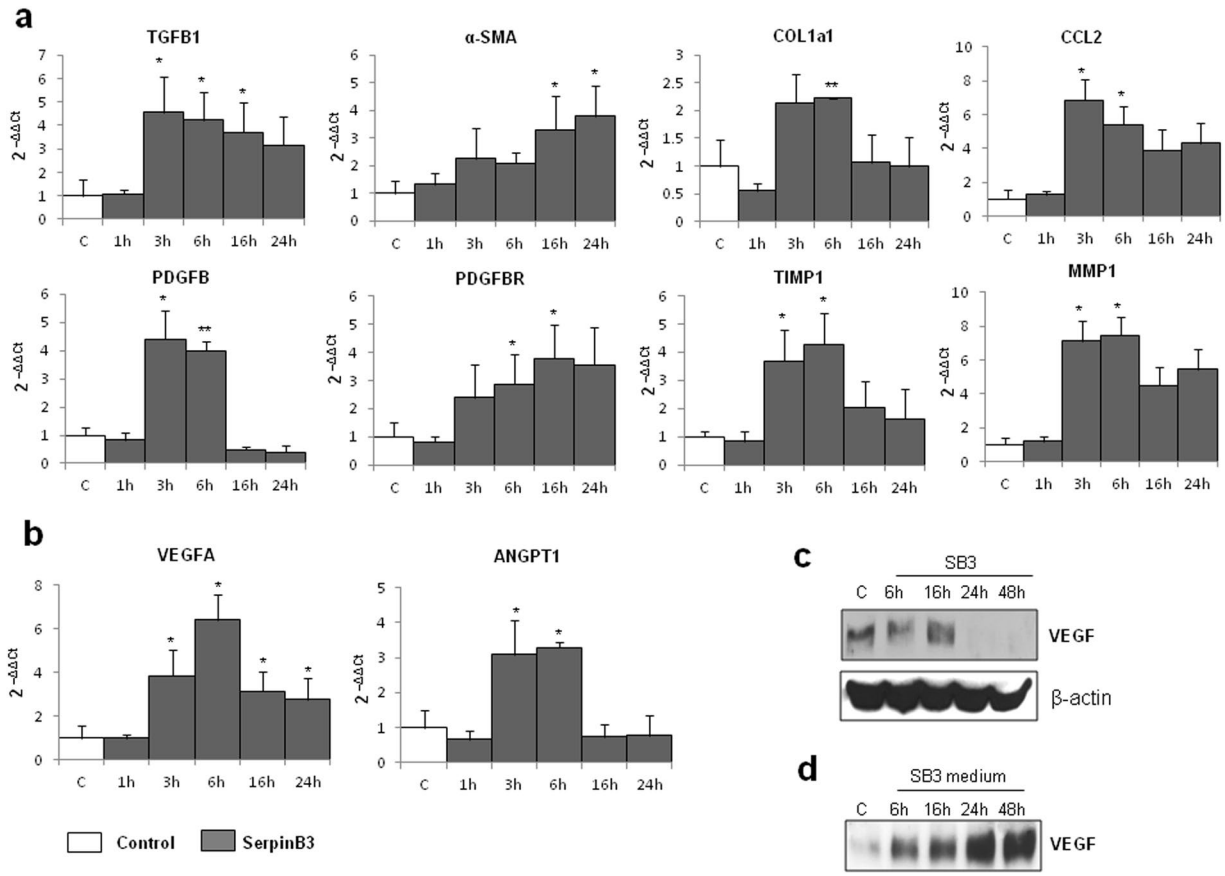

Figure 1. SerpinB3 up-regulates expression of genes involved in liver fibrogenesis in immortalized human HSC (LX2 cells). **(a)** Analysis by quantitative real-time PCR (Q-PCR) of transcript levels of pro-fibrogenic genes, of CCL2 as well as of **(b)** VEGF-A or angiopoietin 1 (Ang-1) in human LX2 cells exposed for the indicated time points to 100 ng/ml human recombinant SerpinB3 (SB3). Data are expressed as means ± SEM of three independent experiments (*$p < 0.05$ or **$p < 0.01$ vs control values). **(c,d)** WB Analysis of VEGF-A protein in total extracts (left panel) and immuno-precipitated VEGF-A protein levels released in the extracellular medium collected at the indicated time points. The cropped gels shows in this Fig. have been run under the same experimental conditions.

and SerpinB3 expression (protein and mRNA level). The same study also reported "*in vitro*" data showing that SerpinB3 over-expression in hepatocyte-derived cells also promoted TGF-β1 release, suggesting that SerpinB3 may contribute to up-regulate TGF-β1 production during chronic injury[12]. The putative pro-fibrogenic action of SerpinB3 has also been evidenced in human idiopathic pulmonary fibrosis[22] and in a murine model of lung fibrosis[23,24]. Nonetheless, the mechanisms by which SerpinB3 may contribute to liver fibrogenesis are still poorly characterized.

In the present study we investigated the putative pro-fibrogenic role of SerpinB3 first by performing experiments "*in vitro*" using human HSC/MFs and then "*in vivo*" taking advantage of two different experimental protocols of chronic liver injury in transgenic mice overexpressing human SerpinB3 in hepatocytes (TG-SB3 mice).

Results

SerpinB3 up-regulates transcription of key pro-fibrogenic genes in human HSC/MFs. The addition of human recombinant SerpinB3 (hrSerpinB3) to cultures of human immortalized activated MF-like cells (LX2 cells) resulted in the up-regulation of the transcripts for key pro-fibrogenic genes, including TGFβ1 (TGFB1), COL1A1, α-SMA, platelet-derived growth factor (PDGF)-B and the related PDGF-β receptor (PDGFBR) subunit, TIMP-1, MMP-1 as well as C-C motif chemokine ligand 2 (CCL2) (Fig. 1a). The activation of these genes was also fully reproduced in primary cultures of human HSC/MFs exposed to hrSerpinB3 (Supplementary Fig. 1a). Furthermore, upon hrSerpinB3 addition, both LX2 cells and primary culture of human HSC/MFs up-regulated the transcription of pro-angiogenic cytokines relevant in sustaining liver fibrogenesis[25,26] such as VEGF-A (VEGFA) and Angiopoietin-1 (Ang-1 or ANGPT1) (Fig. 1b; Supplementary Fig. 2). In HSC/MFs SerpinB3 also stimulated the expression of TIE-2, the gene encoding for the Ang-1 receptor (Supplementary Fig. 2). Interestingly, already 6 hours after hrSerpinB3 addition LX2 cells released VEGF-A protein in the extracellular medium and this effect reached a maximum in the following 24–48 hours (Fig. 1c,d).

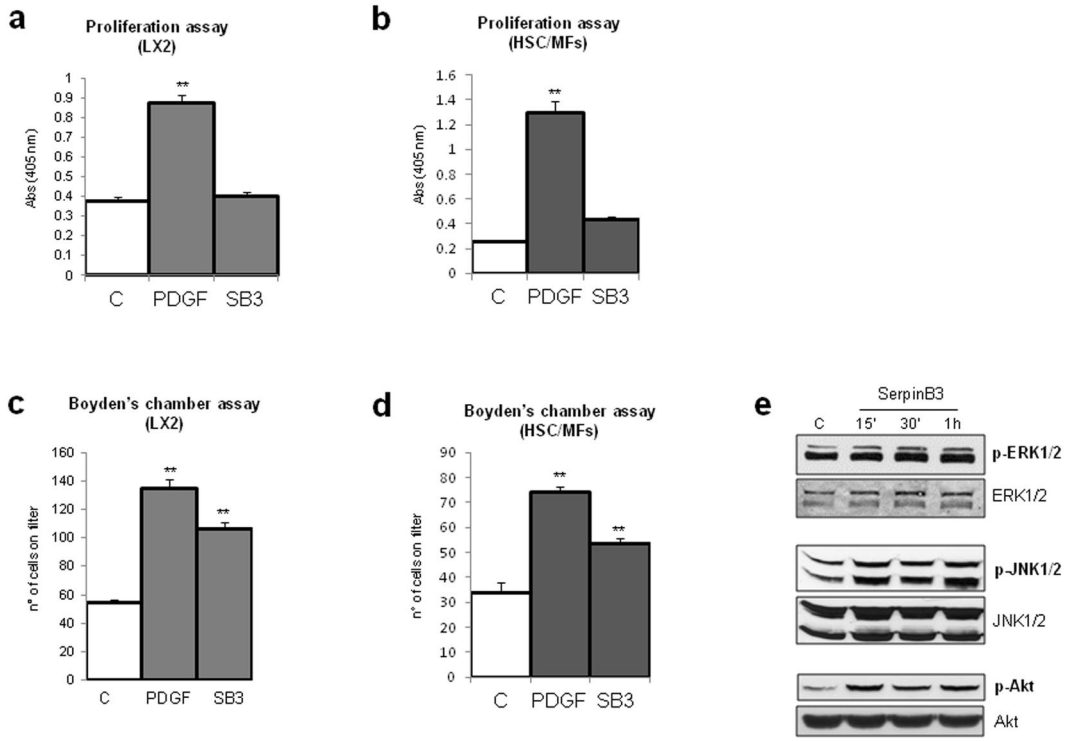

Figure 2. hrSerpinB3 stimulates oriented migration, but not proliferation, in human LX2 cells and human HSC/MFs. **(a,b)** Confluent and 24 hr starved LX2 cells (**a**) or HSC/MFs (**b**) were left untreated or exposed to either hrSerpinB3 (100 ng/ml) or PDGF-BB (10 ng/ml, positive control) for further 48 hrs and then evaluated for proliferation using the crystal violet assay (see Methods). **(c,d)** Analysis of oriented migration in the modified Boyden's chamber assay of LX2 cells (**c**) or HSC/MFs (**d**) exposed or not to either hrSerpinB3 (100 ng/ml) or PDGF-BB (10 ng/ml, positive control). Data in bar graphs are expressed as means ± SEM of three independent experiments (**$p < 0.01$ vs control values). **(e)** Western blot analysis of activation of ERK1/2, JNK1/2 and Akt signaling pathways in LX2 cells exposed to hrSerpinB3 (100 ng/ml). Confluent and 24 hr starved LX2 cells were exposed to SerpinB3 for the indicated time points and activation of pathways was evaluated in total extracts by analysis of levels of phosphorylated versus un-phosphorylated levels of ERK1/2, JNK1/2 and c-Akt. The cropped gels shows in this Fig. have been run under the same experimental conditions.

SerpinB3 induces oriented migration in human HSC/MFs. We next evaluated whether SerpinB3 may modulate proliferation and chemotaxis of activated HSCs. Human LX2 cells as well as human primary HSC/MFs, which responded positively to the potent mitogen and chemoattractant PDGF-BB (used as positive control), failed to show appreciable increase in proliferation when exposed to hrSerpinB3 (Fig. 2a,b). However, both LX2 cells (Fig. 2c) and human HSC/MFs (Fig. 2d) showed oriented migration when exposed to hrSerpinB3 in a modified Boyden's chamber assay. Additional experiments, designed to investigate the chemoattractant action, revealed that LX2 cells exposed to hrSerpinB3 had features in common with those induced by other chemo-attractants on activated HSCs[27]. This included very early activation/phosphorylation of c-Jun NH_2-terminal kinase isoforms −1 and −2 (JNK1/2) and c-Akt, with no relevant change in activation/phosphorylation of extracellular-regulated kinase isoforms 1 and 2 (ERK1/2) (Fig. 2e). In addition, hrSerpinB3 also promoted HSC/MF generation of intracellular ROS that was evident already after 15 min (Fig. 3b). As previously shown for other chemoattractants[27], hrSerpinB3-induced oriented migration was almost abolished by pre-treating HSC/MFs with pharmacological inhibitors of either NADPH-oxidase (apocynin, Diphenyleneiodonium or DPI) or of JNK1/2 and c-Akt (SP600125 or LY294002) (Fig. 3c). Furthermore, apocynin-mediated inhibition of ROS generation by NADPH-oxidase also blocked the activation/phosphorylation of JNK1/2 but not that of c-Akt (Fig. 3d), confirming that the chemotactic action of SerpinB3 mainly involves the same ROS- and JNK1/2-related signals described for other chemoattractants like PDGF-BB, VEGF-A and CCL2 that are active on HSC/MFs[27].

Transgenic mice over-expressing human SerpinB3 in murine hepatocytes develop more liver fibrosis than wild type littermates. As mentioned above, SerpinB3 is virtually undetectable in either human or murine livers, but it is produced in response to liver hypoxia by hepatocytes or hepatic cancer cells in a significant fraction of patients with cirrhosis or hepatocellular carcinoma[19]. In order to mechanistically investigate whether SerpinB3 may operate as a pro-fibrogenic mediator we employed previously characterized transgenic mice that overexpress human SerpinB3 (TG-SB3 mice) in hepatocytes and in lung epithelial cells[24]. The livers of naïve SerpinB3 TG-mice had normal morphology and hepatocytes of naïve SerpinB3 TG-mice as well as of related wild type mice did not differ for the expression of albumin, cytokeratin 18, α-fetoprotein or the

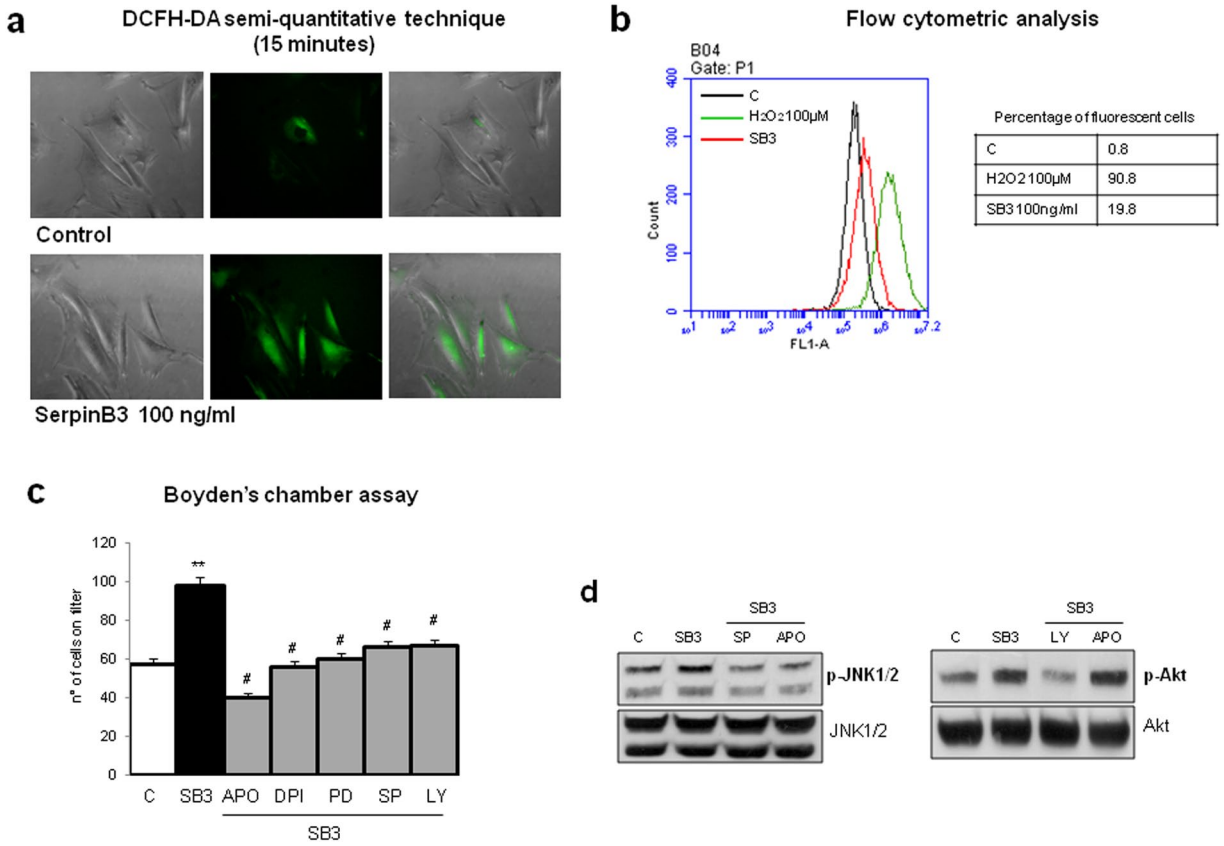

Figure 3. hrSerpinB3-dependent induction of oriented migration relies on intracellular generation of ROS and specific signaling pathways. **(a,b)** Confluent and 24 hr starved LX2 cells were left untreated or exposed to either hrSerpinB3 (100 ng/ml) or PDGF-BB (10 ng/ml, positive control) and then evaluated for early (i.e., 15 minutes) intracellular generation of ROS as shown by DCFH-DA fluorescence (**a**) or flow cytometry analysis (**b**). **(c,d)** Analysis of oriented migration in the modified Boyden's chamber assay (**c**) or Western blot analysis of activation of JNK1/2 and Akt signaling pathways (**d**) of control LX2 cells or LX2 cells exposed to hrSerpinB3 (100 ng/ml) in the presence of pharmacological inhibitors of JNK1/2 (SP, SP600125,) and Akt (LY, LY294002) or in the presence of apocynin (APO) or diphenylen-iodonium (DPI), all added 30 min before addition of hrSerpinB3. Data in bar graphs are expressed as means ± SEM of three independent experiments (*$p < 0.05$ or **$p < 0.01$ vs control values, $^{\#}p < 0.05$ vs related stimulus). Images from Western blot analysis or from evaluation of ROS generation are representative from at least three experiments performed. The cropped gels shows in this Fig. have been run under the same experimental conditions.

ability to store glycogen and were all negative for cytokeratin 7 expression (Supplementary Figure 3). SerpinB3 expression was evident in parenchymal cells (Fig. 4a) and Western blot analysis confirmed the exclusive presence of human SerpinB3 in TG animals (Fig. 4b). This pattern corresponded to that seen in CLD patients where SerpinB3 immunostaining was mainly detectable in hepatocytes (Fig. 4c). In human specimens from HCV cirrhotic patients (METAVIR F4) SerpinB3-positive cells were usually detectable in hypoxic areas, as shown by the concomitant expression of HIF2α and VEGF-A (Fig. 4d).

For evaluating the actual impact of SerpinB3 on the evolution of hepatic fibrosis TG-SB3 mice and corresponding WT littermates were exposed to chronic liver injury. To this aim we used two different experimental murine models: (i) chronic CCl_4 administration, which results in post-necrotic bridging fibrosis resembling pan-lobular/parenchymal fibrosis found in the clinical conditions in which SerpinB3 expression has been best characterized[2, 28, 29]; (ii) mice fed with the MCD diet, that leads to pericellular/perisinusoidal fibrosis similar to that seen in human NAFLD/NASH[2, 28, 29]. Naive TG-SB3 mice had plasma SerpinB3 levels around 2 ng/mL (1.8 ± 0.6 ng/mL) that were enhanced by 5–10 fold in response to chronic liver injury. In particular, CCl_4-treated animals showed circulating SerpinB3 twice as higher than those receiving the MCD diet (21.5 ± 6.7 ng/mL vs 10.5 ± 5.4 ng/mL; $p < 0.001$). In the transgenic animals SerpinB3 over-production did not influence hepatocellular damage, as liver histology and the circulating levels of alanine (ALT) and aspartate (AST) aminotransferases were comparable to those in wild type mice (Fig. 5). On the other hand, TG-SB3 mice receiving CCl_4 or the MCD diet showed a higher hepatic expression of inflammatory markers TNF-α and CD11b (Fig. 5) than the relative wild type animals. In both models of chronic liver injury, collagen Sirius Red staining revealed that the overexpression of SerpinB3 resulted in a marked increase in the extension of fibrosis. In a similar manner the number of α-SMA positive HSC/MFs was significantly higher in TG-SB3 mice exposed to CCl_4 or the MCD

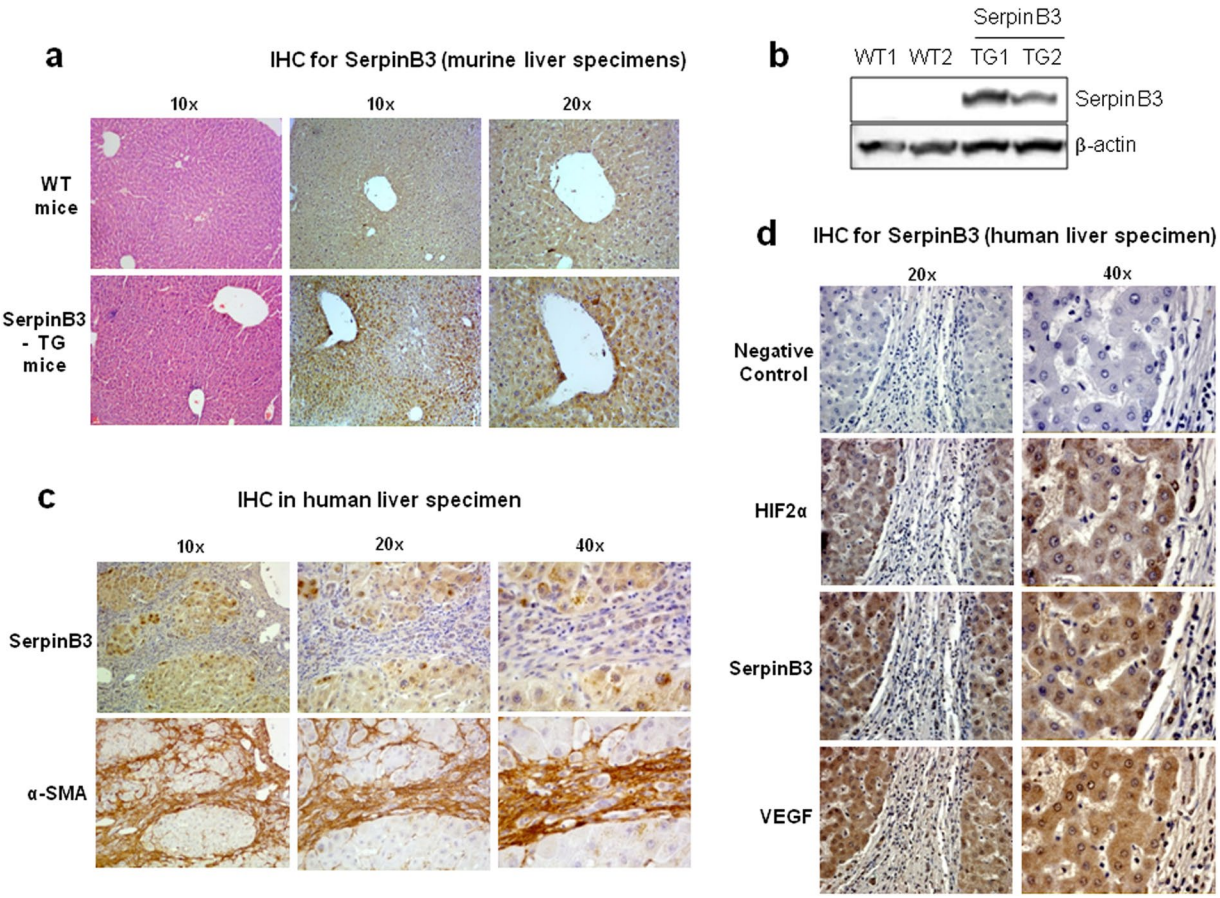

Figure 4. SerpinB3 overexpression in TG mice and human liver specimens from HCV patients. (**a**) Liver morphology (hematoxylin/eosin staining) and immunohistochemistry (IHC) for SerpinB3 expression in the liver of control WT and TG mice. Original magnification as indicated. **(b)** Western blot analysis of SerpinB3 protein levels performed on total liver extracts obtained from either WT and TG mice. Equal loading was monitored by re-blotting membranes for β-actin. The cropped gels shows in this Fig. have been run under the same experimental conditions. Immunohistochemistry analysis for **(c)** SerpinB3 and α-smooth muscle actin (α-SMA) or **(d)** for HIF2α, SerpinB3, and VEGF-A in liver specimens from HCV infected patients (METAVIR F4).

diet than in similarly treated wild-type animals (Figs 6 and 7). Consistently, Q-PCR analysis for a set of genes involved in fibrogenesis demonstrated that in both the experimental models the hepatic transcripts for collagen 1A1 (Col1a1), tissue inhibitor of metalloproteases type 1 (Timp1) and TGFβ1 (Tgfb1) were significantly enhanced in TG-SB3 (Figs 6 and 7). Among TG-SB3 mice receiving CCl_4 and the MCD diet we also observed a significant positive correlation ($r = 0.74$; $p = 0.04$) between the individual levels of circulating SerpinB3 and those of liver collagen 1A1 transcripts. The mRNAs for heme-oxygenase 1 (Hmox-1) and matrix-metalloprotease type 2 (Mmp2) were also elevated in TG-SB3 animals receiving the MCD diet, but not in those exposed to CCl_4 (Figs 6 and 7). Altogether, these data provide *in vitro* and *in vivo* evidence supporting the implication of SerpinB3 in the processes leading to CLD progression to fibrosis.

Discussion

SerpinB3 is virtually undetectable in normal human livers, but several studies have evidenced the presence of SerpinB3 in liver biopsies from patients with CLD, mostly chronic HCV infection, where it has been associated to CLD progression and liver carcinogenesis[13–21]. In fact, in CLD patients SerpinB3 up-regulation correlates with TGF-β1 expression and the extent of hepatic fibrosis[12]. Furthermore, experiments using hepatocyte-derived cell lines over-expressing SerpinB3 have evidenced its role in promoting TGF-β1 production, suggesting SerpinB3 as a putative pro-fibrogenic mediator. More recently, SerpinB3 expression has been reported to be up-regulated by hypoxia through a HIF-2α-dependent and ROS-modulated mechanism[19]. This is a potentially critical issue, since in the last decade liver hypoxia and angiogenesis have been suggested to parallel or even drive the fibrogenic progression of CLDs[6,25,26,29–31]. Literature data relating SerpinB3 with advanced cirrhosis and hepatocellular carcinoma have also evidenced that, beside enhancing TGF-β1 expression, SerpinB3 can induce epithelial-mesenchymal transition (EMT) of hepatoma cancer cells, resulting in their increased invasiveness and proliferation[12,17,19,21]. Indeed, in HCC patients high levels of SerpinB3 were significantly associated with

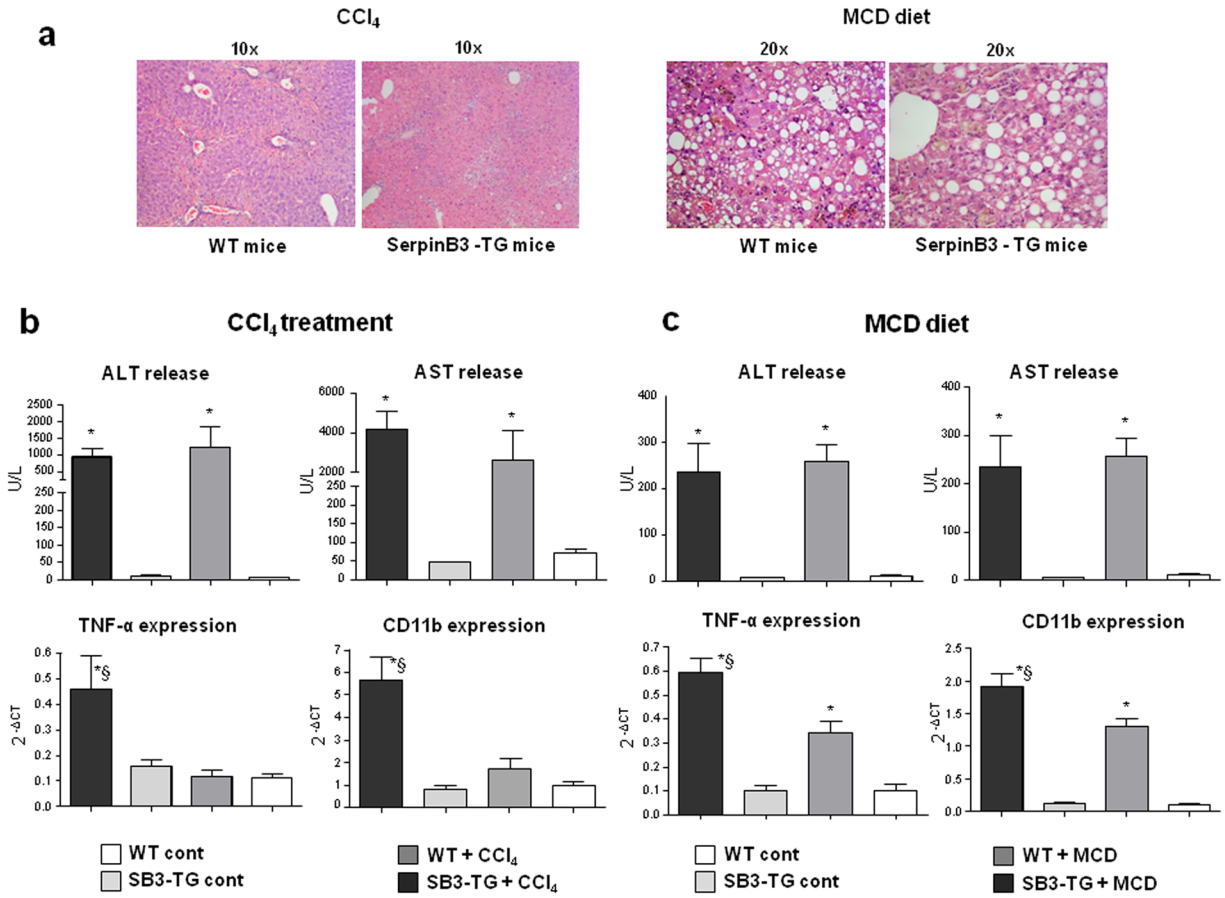

Figure 5. Parenchymal injury and inflammatory markers in SerpinB3 transgenic (SB3-TG) mice and wild type (WT) mice following chronic (10 weeks) CCl_4 administration or 4 weeks feeding with a methionine/choline deficient (MCD) diet. **(a)** Liver morphology in mice exposed to CCl_4 or the MCD diet was evaluated by hematoxylin/eosin staining (magnification 10X). Parenchymal injury, estimated by measuring the circulating levels of alanine (ALT) and aspartate (AST) aminotransferases and liver expression of inflammatory markers TNF-α and CD11c, evaluated by quantitative real-time PCR (Q-PCR), are reported in CCl_4 treated mice **(b)** and in MCD fed mice **(c)**. Data in graphs are expressed as means ± SD (n = 6 mice for each experimental group) (*$p < 0.01$ vs the relative control mice; $^{\S}p < 0.05$ vs WT-mice receiving CCl_4 or the MCD diet).

early tumour recurrence and poor prognosis[21]. However, the involvement of EMT in liver fibrogenesis has been recently challenged, with most researchers suggesting a minor, if any, pro-fibrogenic role for EMT and supporting the concept that MFs originate mainly from activation/trans-differentiation of HSC[4,6,8,9,32]. These considerations have led us to explore the hypothesis that SerpinB3 might directly affect the behaviour of MF-like cells.

Our *in vitro* experiments show that hrSerpinB3 can directly act on activated HSCs by both up-regulating the transcription of critical genes involved in fibrogenesis and stimulating their oriented migration. In these settings, the capacity of the cells to readily respond to hrSerpinB suggests that SerpinB3 may exert its action on a receptor-mediated basis. Furthermore, at difference of other mediators involved in fibrogenesis, SerpinB3 action on human activated HSCs seems quite selective and does not involves cell proliferation. Unfortunately, at present a specific receptor for SerpinB3 has not yet been characterized. Whatever might be the receptor involved, in human HSC/MFs SerpinB3 promotes NADPH-oxidase-dependent generation of intracellular ROS and ROS-related activation of JNK1/2 signalling pathways. These intracellular events have been implicated in both up-regulation of ECM synthesis/remodelling by MFs as well as in the induction of their oriented migration[1–6]. The latter is a relevant issue for these cells in order to align with nascent and more mature fibrotic septa. On this respect, it is noteworthy that the signal pathways leading to SerpinB3-induced oriented migration are identical to those characterized in human HSC/MFs migrating in response to chemo-attractant polypeptides (PDGF-BB, CCL2 and VEGF) or hypoxic conditions[27,30].

Beside the chemotactic action, the exposure of HSC/MFs or LX2 cells to hrSerpinB3 also results in an increased transcription of genes relevant for the fibrogenic progression of CLD along with VEGF-A, Angiopoietin-1 and CCL2, suggesting that SerpinB3 may combine pro-fibrogenic and pro-angiogenic activities. Of interest, the pro-fibrogenic genes stimulated "*in vitro*" by hrSerpinB3 are also up-regulated in response to chronic liver injury in transgenic mice over-expressing human SerpinB3. Accordingly, by using SerpinB3 transgenic mice we observed that the induction of chronic liver injury by repeated exposure to CCl_4, or the

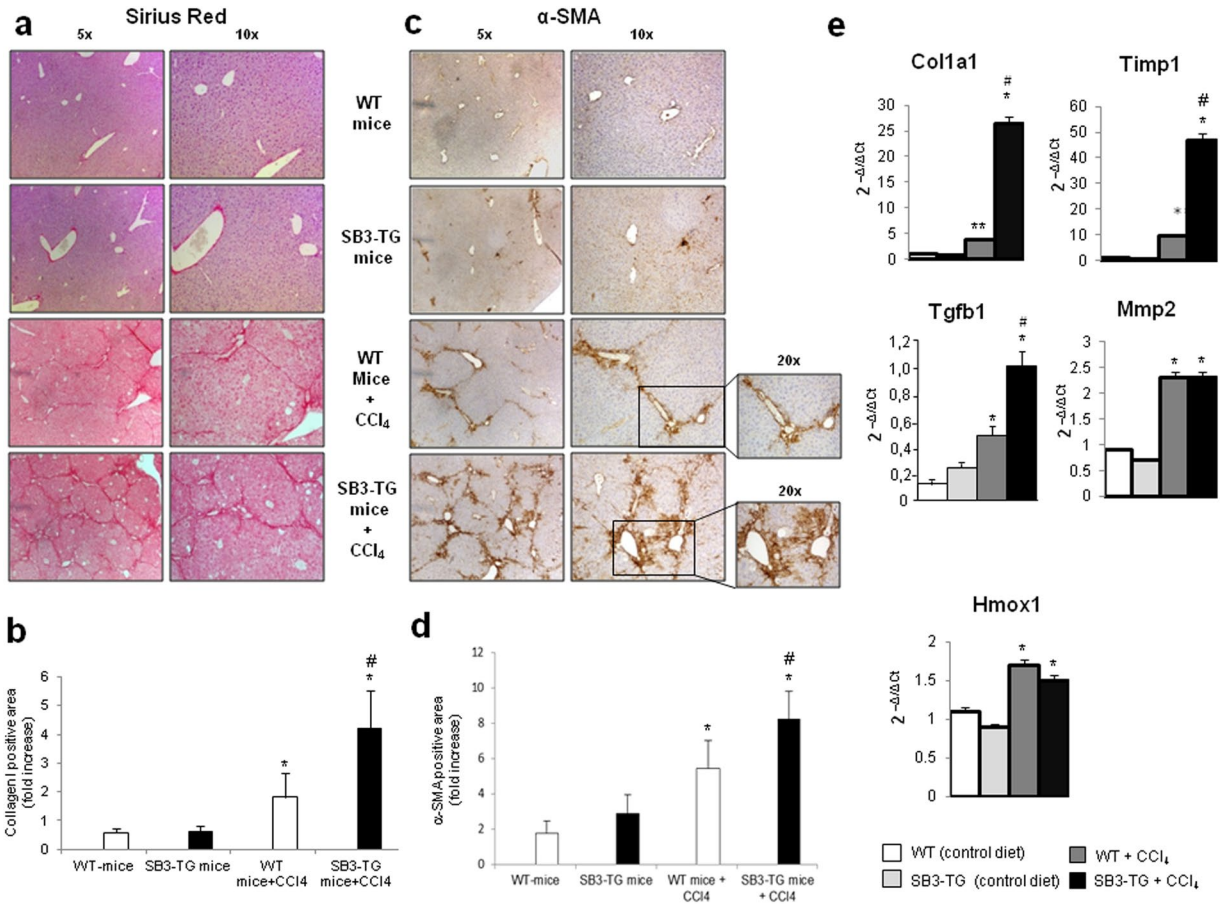

Figure 6. Liver fibrosis "*in vivo*" in SerpinB3 transgenic (TG) mice vs wild type (WT) mice exposed to chronic CCl_4 administration. **(a,c)** Following chronic exposure to CCl_4 (10 weeks) liver fibrosis was morphologically evaluated by Sirius red staining (**a**) or by immunohistochemistry for α-SMA (**c**). Original magnification as indicated. **(b,d)** ImageJ software analysis was performed for both Sirius red staining (**b**) and α-SMA immunohistochemistry analysis (**d**) to evaluate the amount of fibrosis. **(e)** Analysis by quantitative real-time PCR (Q-PCR) of SerpinB3 transcript levels of pro-fibrogenic genes in the different experimental groups. Data in graphs are expressed as means ± SEM (n = 6 mice for any experimental group) (*$p < 0.05$ vs relative control mice; #$p < 0.05$ vs WT-mice treated with CCl_4).

development of steatohepatitis in mice fed the MCD diet leads to a massive stimulation in SerpinB3 production by the hepatocytes and a very significant increase in ECM deposition. This later effect was associated with an increase in the number of α-SMA-positive cells (i.e., MF-like cells) as well as in an enhanced transcription of several pro-fibrogenic genes, including collagen 1A1, TGFβ1, α-SMA, PDGF-B and TIMP1 as compared to WT-mice. This scenario resembles that previously outlined for bleomycin-induced lung fibrosis, in which a direct correlation between SerpinB3 expression, TGFβ1 levels and lung fibrosis was reported[24]. The enhanced deposition of extracellular matrix observed in SerpinB3 transgenic mice exposed to chronic liver injury is independent from the extent of parenchymal injury supporting *in vitro* data concerning direct stimulation of MF-like cells by SerpinB3. Nonetheless, we have observed that hepatic damage in SerpinB3 transgenic mice associates with an enhanced expression of inflammatory markers. At present, it is still unclear whether SerpinB3 can directly sustain the activation of liver inflammatory cells but since chronic inflammation is one of the driving force for the progression of hepatic fibrosis, we cannot exclude that a pro-inflammatory action of SerpinB3 might also contribute to the dramatic increase in liver collagen deposition observed in our experimental settings. By contrast, we do not have *in vitro* or *in vivo* evidence that other non-parenchymal cells, in particular macrophages, may express and release SerpinB3.

A previous study has shown that SerpinB3 expression in the liver is modulated by hypoxia through HIF2α-dependent mechanisms[19]. Based on the knowledge that hypoxic conditions have a pro-fibrogenic and pro-angiogenic role during the progression of CLD[1–6,25,26,29–34], our study points on the possible implication of SerpinB3 among the mediators responsible for the pro-fibrogenic action of hepatic hypoxia.

In conclusion, the present study provides for the first time evidence indicating that SerpinB3 produced by liver parenchymal cells can contribute to the fibrogenic progression of CLDs by stimulating the responses of HSC/MFs.

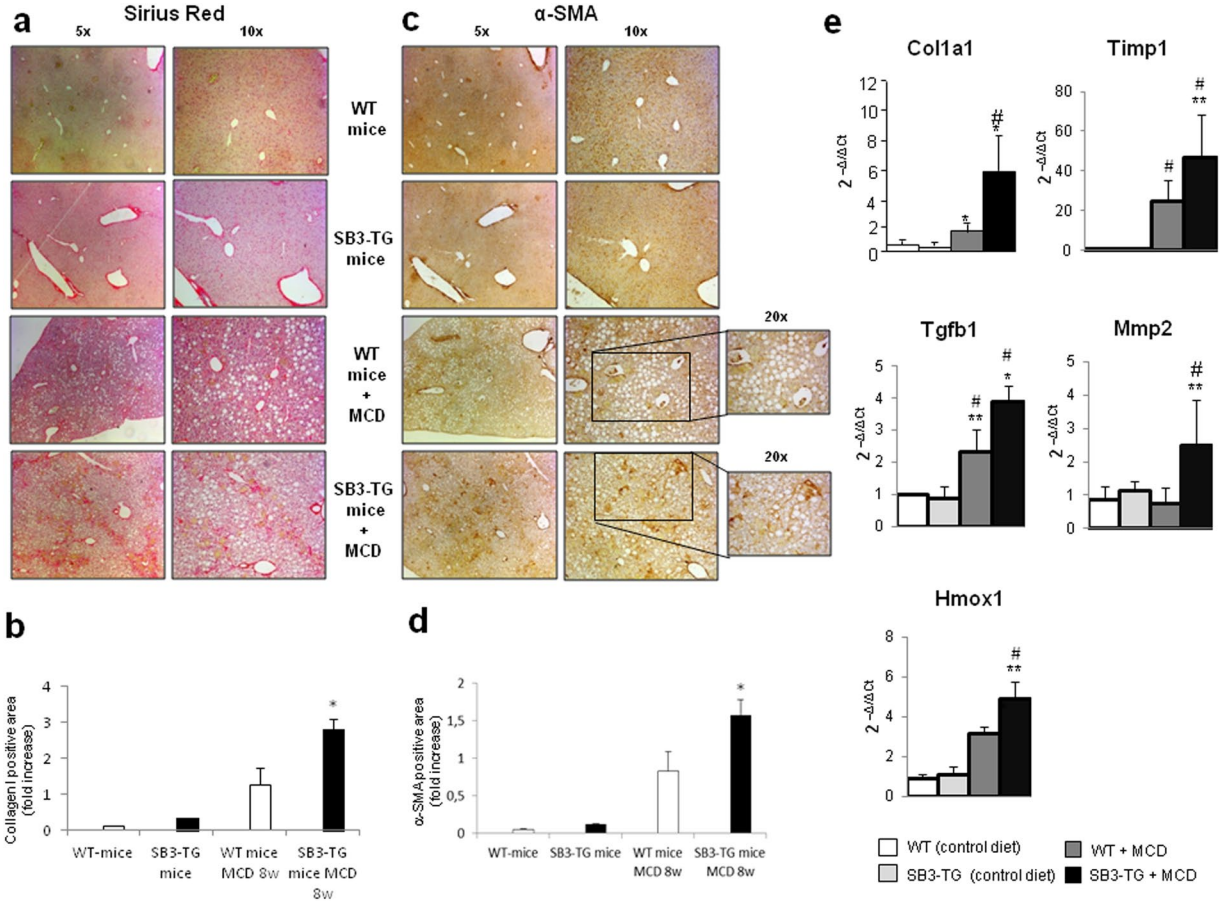

Figure 7. Liver fibrosis "*in vivo*" in SerpinB3 transgenic (SB3-TG) mice vs wild type (WT) mice fed with MCD diet. **(a,c)** Following MCD diet (8 weeks) liver fibrosis was morphologically evaluated by Sirius red staining (**a**) or by immunohistochemistry for α-SMA (**c**). Original magnification as indicated. **(b,d)** ImageJ software analysis was performed for both Sirius red staining (**b**) and α-SMA immunohistochemistry analysis (**d**) to evaluate the amount of fibrosis. (**e**) Analysis by quantitative real-time PCR (Q-PCR) of SerpinB3 transcript levels of pro-fibrogenic genes in the different experimental groups. Data in graphs are expressed as means ± SEM (n = 6 mice for any experimental group) (*$p < 0.05$ or **$p < 0.01$ vs relative control mice; $^{\#}p < 0.05$ vs WT-mice fed with MCD diet). Col1a1: collagen 1A1; Timp1: tissue inhibitor of metalloproteases type 1; Tgfb1: TGFβ1; Hmox-1: heme-oxygenase 1; Mmp2: matrix-metalloprotease type 2.

Materials and Methods

***In vitro* experiments with activated, MF-like, hepatic stellate cells or HepG2 cells.** Human LX2 cells, a model of immortalized and activated, MF-like, human HSC, were kindly provided by Prof. Scott L. Friedman and were cultured in Dulbecco's modified Eagle's medium (Sigma Aldrich Spa, Milan, Italy), supplemented with 10% fetal calf serum and 1% antibiotics. In some experiments we also used human HSC that were isolated from surgical wedge sections of at least three different human livers not suitable for transplantation as previously described[35]. These HSCs, kindly provided by Prof. Fabio Marra, were cultured as previously described[27] and used between passages 4 and 7 when showing a phenotype of fully activated, MF-like HSCs (HSC/MFs), plated to obtain the desired sub-confluence level and then left for 24 hrs in serum-free Iscove's medium to have cells at the lowest level of spontaneous proliferation. In *in vitro* experiments LX2 cells or HSC/MFs were exposed to human recombinant SerpinB3 (hrSerpinB3, 100 ng/ml), obtained as previously described[17].

Detection of intracellular generation of ROS. Detection of ROS generation in cultured cells was performed by either the conversion of 2′,7′-dichlorodihydrofluorescein diacetate (DCFH-DA, 1 μM) into the corresponding fluorescent derivative or by combining DCFH-DA technique and flow cytometric analysis as previously described[19,27]. More details are available in the Supplementary Material section.

Quantitative real-time PCR (Q-PCR). RNA extraction, complementary DNA synthesis, quantitative real-time PCR (Q-PCR) reactions were performed as previously described[19,21]. mRNA levels were measured by Q-PCR, using the SYBR® green method as described[21]. More details and oligonucleotide sequences of primers used for Q-PCR are available in the Supplementary Material section.

Proliferation and Chemotaxis. Proliferation of human LX2 cells or primary HSC/MFs was evaluated by crystal violet proliferation assay by seeding cells in a 96-well plate at a density of 1.5×10^3 cells per well. The cells were incubated in serum-free medium (SFM) for 24 hrs at 100 ng/ml hrSerpinB3 concentration. At the desired time, the medium was removed, and the cells were washed twice with phosphate-buffered saline, fixed with 11% glutaraldehyde; after fixation, cells were washed with H_2O bd and then stained with 0.1% (w/v) crystal violet solution for 10 min. After washing with water, the crystal violet was solubilized with 50 µl of 10% acetic acid solution, and absorbance was measured at 595–650 nm using a microplate reader (SpectraMAX M3; Molecular Devices, Sunnyvale, CA, USA). Chemotaxis of human LX2 cells or HSC/MFs was evaluated by performing the modified Boyden's chamber assay as previously described[27,30].

Experimental fibrosis in SerpinB3 transgenic mice and related wild type mice. The two protocols for experimental fibrosis employed in this study were performed by taking advantage of a model of C57Bl/6 transgenic (TG) mice, fully characterized in previous studies[23,24], that overexpress human SerpinB3 in the liver and lungs. Full details of the experimental protocols on TG-mice and age-matched C57Bl/6 wild type (WT) mice, receiving either i) chronic administration of carbon tetrachloride (CCl_4, Sigma-Aldrich, Milano, Italy) for 10 weeks as described by Wang *et al.*[36] or ii) the methionine- and choline-deficient (MCD) diet up to 8 weeks (as a model of NAFLD/NASH-related fibrosis)[37] are described in the Supplementary Material section. Experiments and protocols, approved by the Animal Ethical Committee of University of Padua and by the Animal Investigation Committee of the Italian Ministry of Health, were performed in accordance with the Helsinki convention and national guidelines and regulations for animal experiments provided by Italian Ministry of Health.

Serological analysis. ALT and AST were determined in serum by laboratory routine assays. Circulating SerpinB3 was evaluated by enzyme-linked immunozymatic assay (SCCA-ELISA, Xeptagen, Marghera, VE, Italy) using biotinylated rabbit anti-human SerpinB3 antibody as previously described[15].

Immunohistochemistry, Sirius Red staining and histo-morphometric analysis. Paraffin liver sections of cirrhotic specimens derived from patients with HCV-related liver cirrhosis (METAVIR F4) were employed. The use of human material conforms to the ethical guidelines of the 1975 Declaration of Helsinki and was approved for this study by the University of Torino Bioethical Committee. Immunostaining procedure[19,27] and Sirius Red staining[38] were as previously described, as detailed in the Supplementary Material section. Quantification of fibrosis in the murine liver was performed by histo-morphometric analysis using a digital camera and a bright field microscope to collect images that were then analyzed by employing the ImageJ software.

Statistical analysis. The data presented are means ± SEM or SD and were obtained from at least three independent experiments. Luminograms and morphological images are representative of at least three experiments with similar results. Statistical analysis for these experiments was performed by Student's t-test or one way ANOVA with Kruskal-Wallis correction for multiple analysis. Significance was taken at $p < 0.05$.

References

1. Friedman, S. Mechanisms of hepatic fibrogenesis. *Gastroenterology* **134**, 1655–1669 (2008).
2. Parola, M., Marra, F. & Pinzani, M. Myofibroblast - like cells and liver fibrogenesis: emerging concepts in a rapidly moving scenario. *Mol Aspects Med* **29**, 58–66 (2008).
3. Wells, R. G. The portal fibroblast: not just a poor man's stellate cell. *Gastroenterology* **147**, 41–47 (2014).
4. Zhang, D. Y. & Friedman, S. Fibrosis-dependent mechanisms of hepatocarcinogenesis. *Hepatology* **56**, 769–775 (2012).
5. Rosselli, M., MacNaughtan, J., Jalan, R. & Pinzani, M. Beyond scoring: a modern interpretation of disease progression in chronic liver disease. *Gut* **62**, 1234–1241 (2013).
6. Novo, E. *et al*. Cellular and molecular mechanisms in liver fibrogenesis. *Arch Biochem Biophys* **548**, 20–37 (2014).
7. Marra, F. & Tacke, F. Roles for chemokines in liver disease. *Gastroenterology* **147**, 577–594 (2014).
8. Mederacke, I. *et al*. Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nat Commun* **4**, 2823 (2013).
9. Wells, R. G. & Schwabe, R. Origin and function of myofibroblasts in the liver. *Semin Liver Dis* **35**, 97–106 (2015).
10. Silverman, G. A. *et al*. The serpins are an expanding superfamily of structurally similar but functionally diverse proteins. *J Biol Chem* **276**, 33293–33296 (2001).
11. Gettins, P. G. Serpin structure, mechanism, and function. *Chem Rev* **102**, 4751–4804 (2002).
12. Turato, C. *et al*. SERPINB3 modulates TGF-β expression in chronic liver disease. *Lab Invest* **90**, 1016–1023 (2010).
13. Biasiolo, A. *et al*. Monitoring SCCA-IgM complexes in serum predicts liver disease progression in patients with chronic hepatitis. *J Viral Hep* **15**, 246–249 (2008).
14. Pontisso, P. *et al*. Overexpression of squamous cell carcinoma antigen variants in hepatocellular carcinoma. *Br J Cancer* **90**, 833–837 (2004).
15. Beneduce, L. *et al*. Squamous cell carcinoma antigen immunoglobulin M complexes as novel biomarkers for hepatocellular carcinoma. *Cancer* **103**, 2558–2565 (2005).
16. Guido, M. *et al*. Squamous cell carcinoma antigen in human liver carcinogenesis. *J Clin Pathol* **61**, 445–447 (2008).
17. Quarta, S. *et al*. SERPINB3 induces epithelial-mesenchymal transition. *J Pathol* **221**, 343–356 (2010).
18. Pontisso, P. Role of SERPINB3 in hepatocellular carcinoma. *Ann Hepatol* **13**, 722–727 (2014).
19. Cannito, S. *et al*. Hypoxia up-regulates SERPINB3 through HIF-2α in liver cancer cells. *Oncotarget* **6**, 2206–2221 (2015).
20. Turato, C. *et al*. Over-expression of SERPINB3 in hepatoblastoma: a possible insight into the genesis of this tumour? *Eur J Cancer* **48**, 1219–1226 (2012).
21. Turato, C. *et al*. SERPINB3 is associated with TGF-β1 and cytoplasmic β-catenin expression in hepatocellular carcinomas with poor prognosis. *Br J Cancer* **110**, 2708–2715 (2014).
22. Calabrese, F. *et al*. Overexpression of squamous cell carcinoma antigen in idiopathic pulmonary fibrosis: clinicopathological correlations. *Thorax* **63**, 795–802 (2008).
23. Villano, G. *et al*. Role of squamous cell carcinoma antigen-1 on liver cells after partial hepatectomy in transgenic mice. *Int J Mol Med* **25**, 137–143 (2010).
24. Lunardi, F. *et al*. Overexpression of SERPIN B3 promotes epithelial proliferation and lung fibrosis in mice. *Lab Invest* **91**, 945–954 (2011).

25. Fernández, M. *et al*. Angiogenesis in liver diseases. *J Hepatol* **50**, 604–620 (2009).
26. Rosmorduc, O. & Housset, C. Hypoxia: a link between fibrogenesis, angiogenesis, and carcinogenesis in liver disease. *Semin Liver Dis* **30**, 258–270 (2010).
27. Novo, E. *et al*. Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. *J Hepatol* **54**, 964–974 (2011).
28. Starkel, P. & Leclerq, I. A. Animal models for the study of liver fibrosis. *Best Practice & Research Clinical Gastroenterology* **25**, 319–333 (2011).
29. Popov, Y. & Schuppan, D. Targeting liver fibrosis: strategies for development and validation of antifibrotic therapies. *Hepatology* **50**, 1294–1306 (2009).
30. Novo, E. *et al*. The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells. *J Pathol* **226**, 588–597 (2012).
31. Nath, B. & Szabo, G. Hypoxia and hypoxia inducible factors: diverse roles in liver diseases. *Hepatology* **55**, 622–633 (2012).
32. Xie, G. & Diehl, A. M. Evidence for and against epithelial-to-mesenchymal transition in the liver. *Am J Physiol Gastrointest Liver Physiol* **305**, G881–890 (2013).
33. Aleffi, S. *et al*. Proangiogenic cytokines as hypoxia-dependent factors stimulating migration of human hepatic stellate cells. *Hepatology* **42**, 1339–1348 (2005).
34. Cannito, S. *et al*. Hypoxia, hypoxia-inducible factors and fibrogenesis in chronic liver diseases. *Histol Histopathol* **29**, 33–44 (2014).
35. Casini, A. *et al*. Regulation of extracellular matrix synthesis by transforming growth factor beta 1 in human fat-storing cells. *Gastroenterology* **105**, 245–253 (1993).
36. Wang, L. *et al*. Effects of retinoic acid on the development of liver fibrosis produced by carbon tetrachloride in mice. *Biochimica et Biophysica Acta* **1772**, 66–71 (2007).
37. Locatelli, I. *et al*. Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. *Hepatology* **60**, 531–544 (2014).
38. Galastri, S. *et al*. Lack of CC chemokine ligand 2 differentially affects inflammation and fibrosis according to the genetic background in a murine model of steatohepatitis. *Clin Sci* **123**, 459–471 (2012).

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Author Contributions

E.N., G.V., P.P. and M.P. conceived the study; E.N., G.V., C.T., S.C., C.P., C.B., A.B., S.Q., E.M., C.B., A.M., E.D. and S.S. carried out experiments and were involved in data acquisition and analysis; E.N., G.M., M.P.L., E.A., M.P. and P.P. supervised experiments, were involved in data interpretation and manuscript editing. All Authors were involved in writing the paper and had final approval of the submitted manuscript.

Additional Information

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2.1 Supplementary information related to PAPER n.2

"SerpinB3 Promotes Pro-fibrogenic Responses in Activated Hepatic Stellate Cells."

Erica Novo, Gianmarco Villano, Cristian Turato, Stefania Cannito, Claudia Paternostro, Chiara Busletta, Alessandra Biasiolo, Santina Quarta, Elisabetta Morello, Claudia Bocca, Antonella Miglietta, Ezio David, Salvatore Sutti, Mario Plebani, Emanuele Albano, Maurizio Parola & Patrizia Pontisso.

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Supplementary information

Manuscript: SerpinB3 Promotes Pro-fibrogenic Responses in Activated Hepatic Stellate Cells

By: **Erica Novo**[1†], **Gianmarco Villano**[2†], Cristian Turato[3], Stefania Cannito[1], Claudia Paternostro[1], Chiara Busletta[1], Alessandra Biasiolo[2], Santina Quarta[2], Elisabetta Morello[1], Claudia Bocca[1], Antonella Miglietta[1], Ezio David[4], Salvatore Sutti[5], Mario Plebani[2], Emanuele Albano[5], Maurizio Parola[1]*, Patrizia Pontisso[2].

[1]Dept. Clinical and Biological Sciences, Unit of Experimental Medicine & Clinical Pathology, University of Torino, Italy; [2] Dept. Medicine, University of Padova, Italy; [3] Veneto Institute of Oncology IOV – IRCCS, Padua, Italy; [4]Pathology Unit, Ospedale S. Giovanni Battista, Torino, Italy; [5]Dept. of Health Sciences, University"Amedeo Avogadro"of East Piedmont, Novara, Italy.

Supplementary Materials and Methods

Materials

Enhanced chemiluminescence (ECL) reagents, nitrocellulose membranes (Hybond-C extra) were from Amersham Pharmacia Biotech Inc. (Piscataway, NJ, USA). Monoclonal antibody against SerpinB3 (sc-21767), p-ERK1/2 (sc-7383) polyclonal antibody for ERK1/2 (sc-292838), p-Akt1/2/3 (sc-7985-R), Akt1/2/3 (sc-8312), VEGF (sc-152) and HIF-1α (sc-10790) were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Polyclonal antibody for p-JNK and JNK1/2 were from Cell Signaling Technology (Massachusetts, USA) and polyclonal antibody for HIF-2α was from Novus Biologicals (Cambridge, UK). SP600125 and LY294002 were from Calbiochem (La Jolla, CA, USA). Monoclonal antibodies for β-actin and all other reagents of analytical grade were from Sigma Chemical Co (Sigma Aldrich Spa, Milan, Italy), Boyden's chambers were from Neuro Probe, Inc. (MD, USA). Human recombinant SerpinB3 (hrSerpinB3) was produced in our laboratory, as previously described [1].

Experimental fibrosis in SerpinB3 transgenic mice

The two protocols for experimental fibrosis were carried out by taking advantage of the murine model of C57BL/6 transgenic mice (TG) that overexpress in the liver and lungs human SerpinB3, a transgenic mouse model which has been fully characterized in a previous study [2]. Each of the experimental protocol was performed on 8 TG mice 12-14 weeks old, as well as on an equal number of age-matched C57BL/6 wild type (WT) mice that were used as controls. Mice were submitted then to the following two protocols of chronic liver injury.

1. Chronic carbon tetrachloride (CCl4) administration. Protocol of chronic liver injury as described by Wang et al. [3] requiring chronic administration of the hepatotoxin carbon tetrachloride (CCl_4,

Sigma-Aldrich, Milano, Italy) for 10 weeks. Briefly, mice received intraperitoneally a CCl_4 dose of 50 µl/100 g body weight, dissolved in olive oil (20%), twice a week for 10 weeks [3]. The control group (6 TG and 6 WT) received injections of olive oil vehicle twice a week for 10 weeks. All mice were sacrificed 24 h after the last dose of treatment.

2. Methionine - choline deficient diet (MCD dietary model). Protocol of chronic liver injury reproducing non-alcoholic fatty liver disease (NAFLD) progressing to non-alcoholic steatohepatitis (NASH) and fibrosis [4]. Briefly, mice were fed for 8 weeks with the MCD diet or control diet (Laboratori Dottori Piccioni, Gessate, Italy) as previously described [5].

In all murine experiments, TG and WT mice were kept under specific pathogen-free conditions and maintained with free access to pellet food and water at the Animal Care Facility of the Experimental Surgery Center, University of Padua, Italy. Liver samples were obtained and either immediately used/processed for molecular biology and morphological purposes or immediately frozen and thereafter maintained at -80°C for further analyses.

Detection of intracellular generation of ROS

Cultured cells, seeded in 12-well culture plates (10^5 cells/well), were either exposed to hrSerpinB3 100ng/ml or treated or not with 100µM H_2O_2, used as positive control, for 15 minutes. ROS generation was detected as the conversion of 2',7'-dichlorodihydrofluorescein diacetate (DCFH-DA, 1 µM) into the corresponding fluorescent derivative. Cells were observed and photographed under a Zeiss fluorescence microscope as previously described [6,7]. ROS generation was also detected by combining DCFH-DA technique and flow cytometric analysis [6]. Cells were seeded in P35 dishes ($5x10^5$ cells/dish), cultured for 24 hrs and treated with DCFH-DA 1 µM for 15 minutes to 37°C and then exposed to 100 ng/ml hrSerpinB3 or 100 µM H2O2 for 15

minutes. Cells were rapidly washed with PBS, collected by trypsinization and re-suspended in PBS for analysis. Detection of DCF green fluorescence (FL1) was performed on at least 5,000 cells per sample with a FACScan equipped with a 488 nm argon laser using the CellQuest software (BectonDickinson, Milano, Italy). The peak of FL1 intensity of DCFH-DA-stained control cells was set to channel 101 and retained for all measurements.

Immunohistochemistry, Sirius Red staining and other stainings

Immunostaining procedure was as previously described [6,8]. Briefly, paraffin sections (2 µm thick), mounted on poly-L-lysine coated slides, were incubated with the following antibodies: monoclonal antibody against SerpinB3 (Santa Cruz Biotechnology, CA, USA; dilution 1:50), monoclonal antibody against α-SMA (Sigma Aldrich Spa, Milan, Italy), polyclonal antibodies (AbCam, Cambridge, UK) against cytokeratin 18 (dilution 1:800), cytokeratin 7 (dil. 1:8000) or murine albumin (dil. 1:200) or polyclonal antibody against α-fetoprotein (DAKO, Glostrup, Denmark; dil. 1:800). After blocking endogenous peroxidase activity with 3% hydrogen peroxide and performing microwave antigen retrieval, primary antibodies were labeled by using EnVision, HRP-labeled System (DAKO, Glostrup, Denmark) and visualized by 3'-diaminobenzidine substrate. For negative controls the primary antibodies were replaced by isotype- and concentrations-matched irrelevant antibody. Sirius Red stainings were performed by a slight modification of the procedure previously described [9] consisting in a rapid immersion of slides in diluted hematoxylin in order to obtain nuclear counterstain of liver sections. Ten randomly selected fields (x10 magnification) of picrosirius red-stained liver sections (3-4 µm thick) were analysed in three sections of each animal at a final magnification of x100. Quantification of fibrosis was performed by histomorphometric analysis using a digital camera and a bright field microscope to collect images that were then analysed by employing the ImageJ software. Conventional histological

stainings (Hematoxylin and Eosin, PAS stain for glycogen) were performed on paraffin sections (2 μm thick).

Western Blot analysis

Total cell lysates or nuclear vs cytosolic extracts, obtained as described [6,7], were subjected to sodium dodecyl sulfate-polyacrylamide gel-electrophoresis on 12%, 10% or 7.5% acrylamide gels, incubated with desired primary antibodies, then with peroxidase-conjugated anti-mouse or anti-rabbit immunoglobulins in Tris-buffered saline-Tween containing 2% (w/v) non-fat dry milk and finally developed with the ECL reagents according to manufacturer's instructions. Sample loading was evaluated by reblotting the same membrane with the un-phosphorylated form of protein or with β-actin antibody.

Quantitative real-time PCR (Q-PCR)

RNA extraction, complementary DNA synthesis, quantitative real-time PCR (Q-PCR) reactions were performed as previously described [6,10]. mRNA levels were measured by Q-PCR, using the SYBR® green method as described [10]. The amplification mix was prepared using iTaq Universal Syber Green SuperMix (Biorad Laboratories, Berkeley, CA) following manufacturer's instructions and realtime PCR was performed using Miniopticon ThermoCycler Instrument (Biorad Laboratories, Berkeley, CA). Oligonucleotide sequence of primers used for RT-PCR were:

a) Mouse genes:

Col1a1	sense: 5'-GGGCAAGACAGTCATCGAAT,	reverse: GGTGGAGGGAGTTTACACGA;
Tgfb1	sense: TTGCTTCAGCTCCACAGAGA,	reverse: TGGTTGTAGAGGGCAAGGAC;
Pdgfa	sense: ACCAGGACGGTCATTTACGA,	reverse: GGTGTTACAACAGCCAGTGC;
Pdgfb	sense: CCAGATCTCTCGGAACCTCA,	reverse: GGCTTCTTTCGCACAATCTC;

Timp1	sense: ATGCCCACAAGTCCCAGAAC,	reverse: TACGCCAGGGAACCAAGAAG;
Mmp2	sense: CCAACTACAACTTCTTCCCCC,	reverse: CGAGCAAAAGCATCATCCAC;
Hmox1	sense: CACGCATATACCCGCTACCT,	reverse: AAGGCGGTCTTAGCCTCTTC;

b) Human genes:

TGFB1	sense: GGGACTATCCACCTGCAAGA,	reverse: CCTCCTTGGCGTAGTAGTCG;
α-SMA	sense: ACCCACAATGTCCCCATCTA,	reverse: GAAGGAATAGCCACGCTCAG;
COL1A1	sense: GTGCTAAAGGTGCCAATGGT,	reverse: ACCAGGTTCACCGCTGTTAC;
CCL2	sense: CCCCAGTCACCTGCTGTTAT,	reverse: AGATCTCCTTGGCCACAATG;
PDGFB	sense: TCCCGAGGAGCTTTATGAGA,	reverse: GGGTCATGTTCAGGTCCAAC;
PDGFBR	sense: GGTGACACTGCACGAGAAGA,	reverse: CAATGGTGGTTTTGCAGATG;
TIMP1	sense: AATTCCGACCTCGTCATCAG,	reverse: TGCAGTTTTCCAGCAATGAG;
MMP1	sense: AGGTCTCTGAGGGTCAAGCA,	reverse: CTGGTTGAAAAGCATGAGCA;
HMOX1	sense: ATGACACCAAGGACCAGAGC	reverse: GTGTAAGGACCCATCGGAGA.

Gliceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal reference and co-amplified with target samples using identical Q-PCR conditions. Samples were run in triplicate and mRNA expression was generated for each sample. Specificity of the amplified PCR products was determined by melting curve analysis and confirmed by agarose gel electrophoresis.

In order to evaluate murine transcripts for TNFα, CD11b and β-actin liver RNA was retro-transcribed with High Capacity cDNA Reverse Transcription Kit (Applied Biosystems Italia, Monza, Italy). RT-PCR for these transcripts was performed in a Techne TC-312 thermacycler (TecneInc, Burlington NJ, USA) using TaqMan Gene Expression Master Mix and TaqMan Gene Expression probes for indicated murine genes (Applied Biosystems Italia, Monza, Italy) as previously described by some of us (5).

References for Supplementary Materials and Methods

1. Turato, C., *et al.* SERPINB3 modulates TGF-β expression in chronic liver disease. Lab Invest 90, 1016-1023 (2010).

2. Lunardi, F., *et al.* Overexpression of SERPIN B3 promotes epithelial proliferation and lung fibrosis in mice. Lab Invest 91**,** 945-954 (2011).

3. Wang, L., *et al.* Effects of retinoic acid on the development of liver fibrosis produced by carbon tetrachloride in mice. Biochimica et Biophysica Acta 1772**,** 66-71 (2007).

4. Hebbard, S. & George, J. Animal models of nonalcoholic fatty liver disease. Nat Rev Gastroenterol Hepatol 8**,** 35-44 (2011).

5. Locatelli, I., *et al.* Endogenous annexin A1 is a novel protective determinant in nonalcoholic steatohepatitis in mice. Hepatology 60**,** 531-544 (2014).

6. Cannito, S., *et al.* Hypoxia up-regulates SERPINB3 through HIF-2α in liver cancer cells. Oncotarget 6, 2206-2221 (2015).

7. Novo, E., *et al.* The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells. J Pathol 226, 588-597 (2012).

8. Novo, E., *et al.* Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. J Hepatol 54, 964-974 (2011).

9. Galastri, S., *et al.* Lack of CC chemokine ligand 2 differentially affects inflammation and fibrosis according to the genetic background in a murine model of steatohepatitis. Clin Sci 123, 459-471 (2012).

10. Turato, C., *et al.* SERPINB3 is associated with TGF-β1 and cytoplasmic β-catenin expression in hepatocellular carcinomas with poor prognosis. Br J Cancer **110**, 2708-2715 (2014).

Supplementary Figures

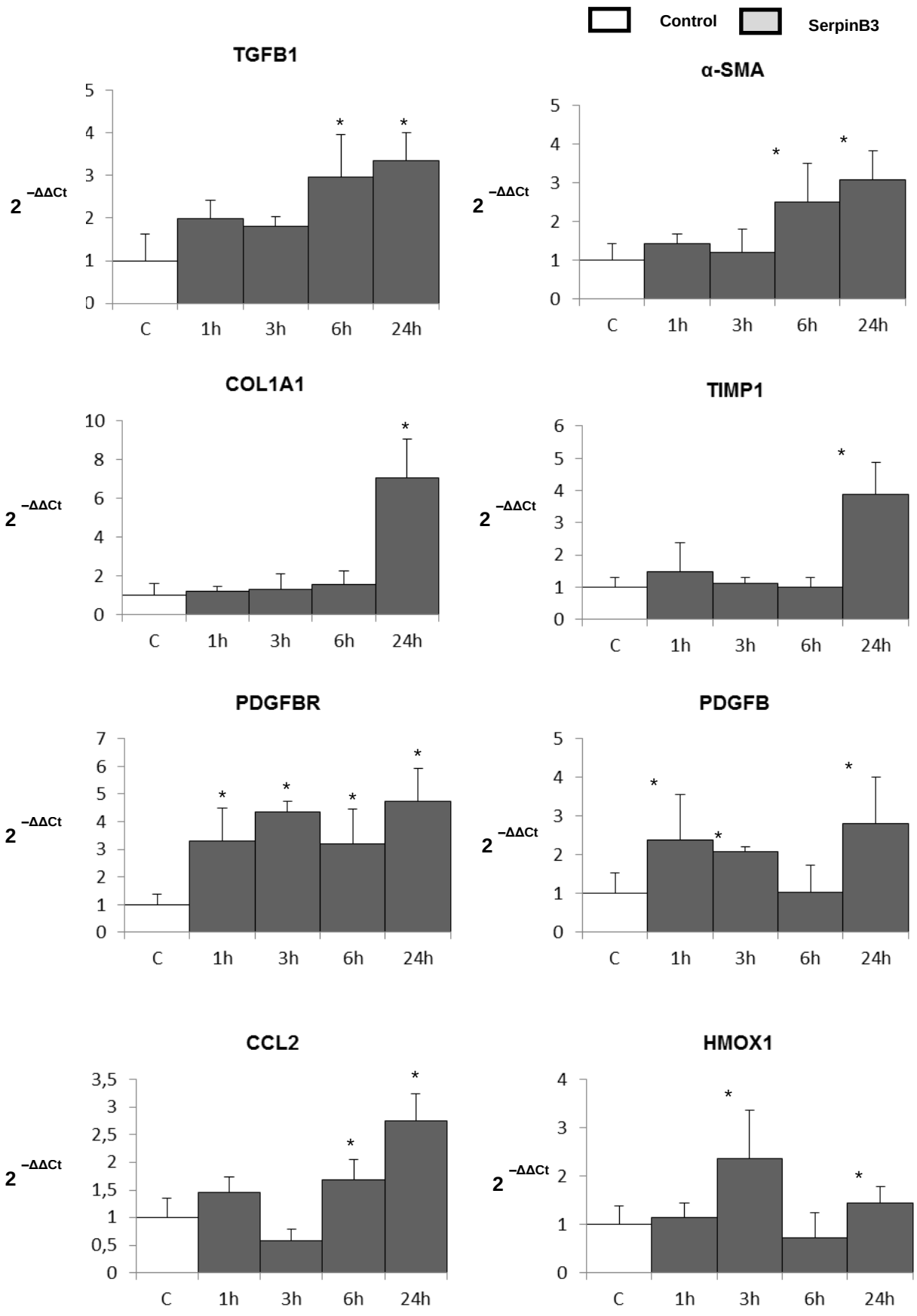
Legend of Supplementary Figures

Supplementary Figure S 1. SerpinB3 up-regulates transcription of critical genes involved in fibrogenesis or inflammation in cultures of human HSC/MFs. Analysis by quantitative real-time PCR (Q-PCR) of transcript levels of the indicated pro-fibrogenic genes as well as of MCP-1 or CCL2 in human HSC/MFs exposed for the indicated time points to 100ng/ml human recombinant SerpinB3 (SB3). Data are expressed as means ± SEM of three independent experiments (*p< 0.05 or **p< 0.01 vs control values).

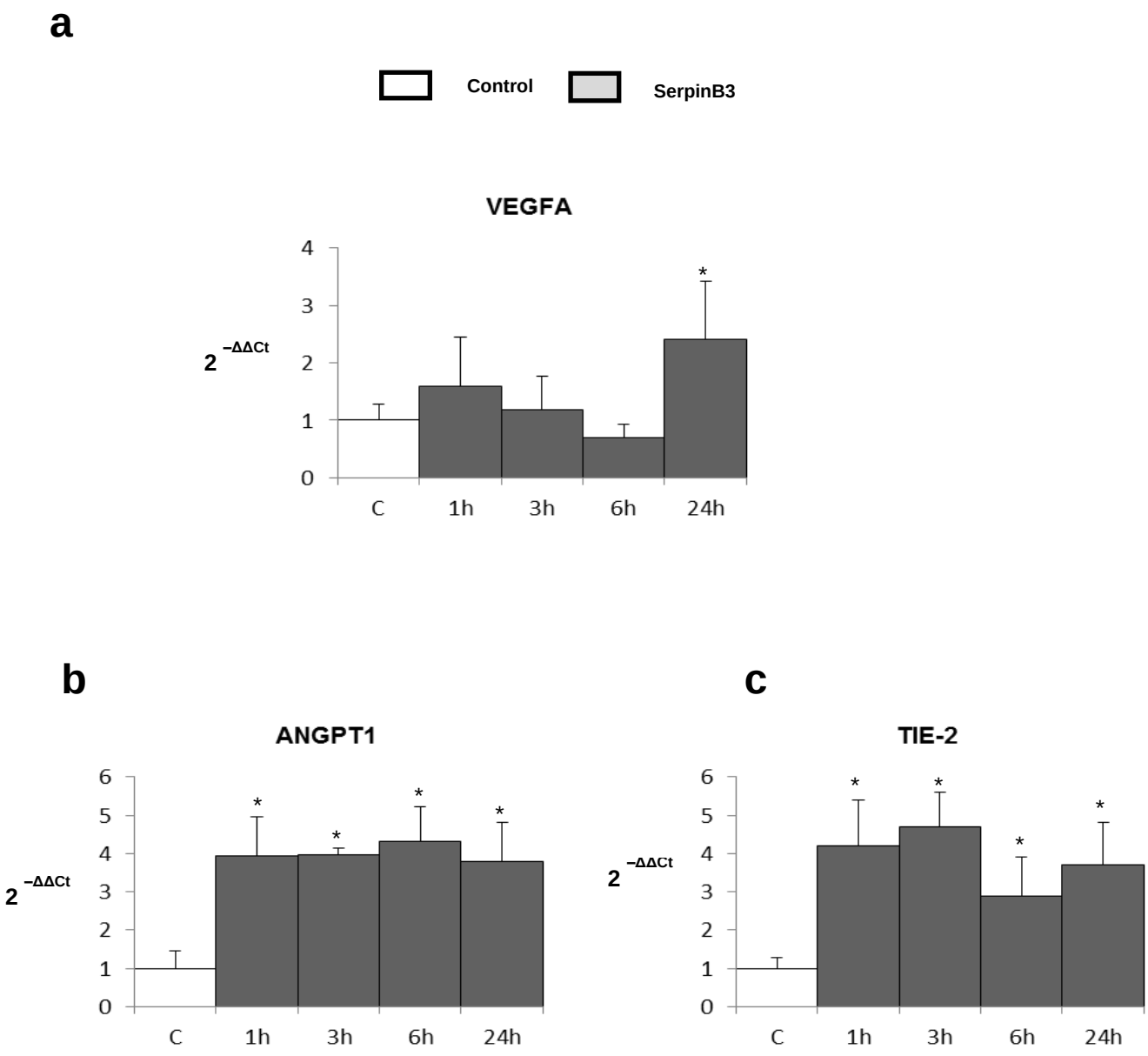
Supplementary Figure S 2. hrSerpinB3 up-regulates transcription of critical genes involved in angiogenesis in cultures of human HSC/MFs. Analysis by quantitative real-time PCR (Q-PCR) of transcript levels of the indicated genes in human HSC/MFs exposed for the indicated time points to 100ng/ml human recombinant SerpinB3 (SB3). Data are expressed as means ± SEM of three independent experiments (*p< 0.05 or **p< 0.01 vs control values).

Supplementary Figure S 3. Morphological comparison between liver specimens from TG-SB3 mice and related wild type mice. Immuno-histochemistry analysis has been performed to investigate hepatocyte expression of murine albumin, α-fetoprotein and cytokeratin 18 (CK-18). Positive stain for cytokeratin 7 (CK-7) was limited to cells of bile ducts or to rare hepatic progenitor cells around portal tracts. Staining of hepatocyte glycogen stores was performed using the standard PAS (periodic acid of Schiff) technique. Magnification as indicated (20x or 40 x).

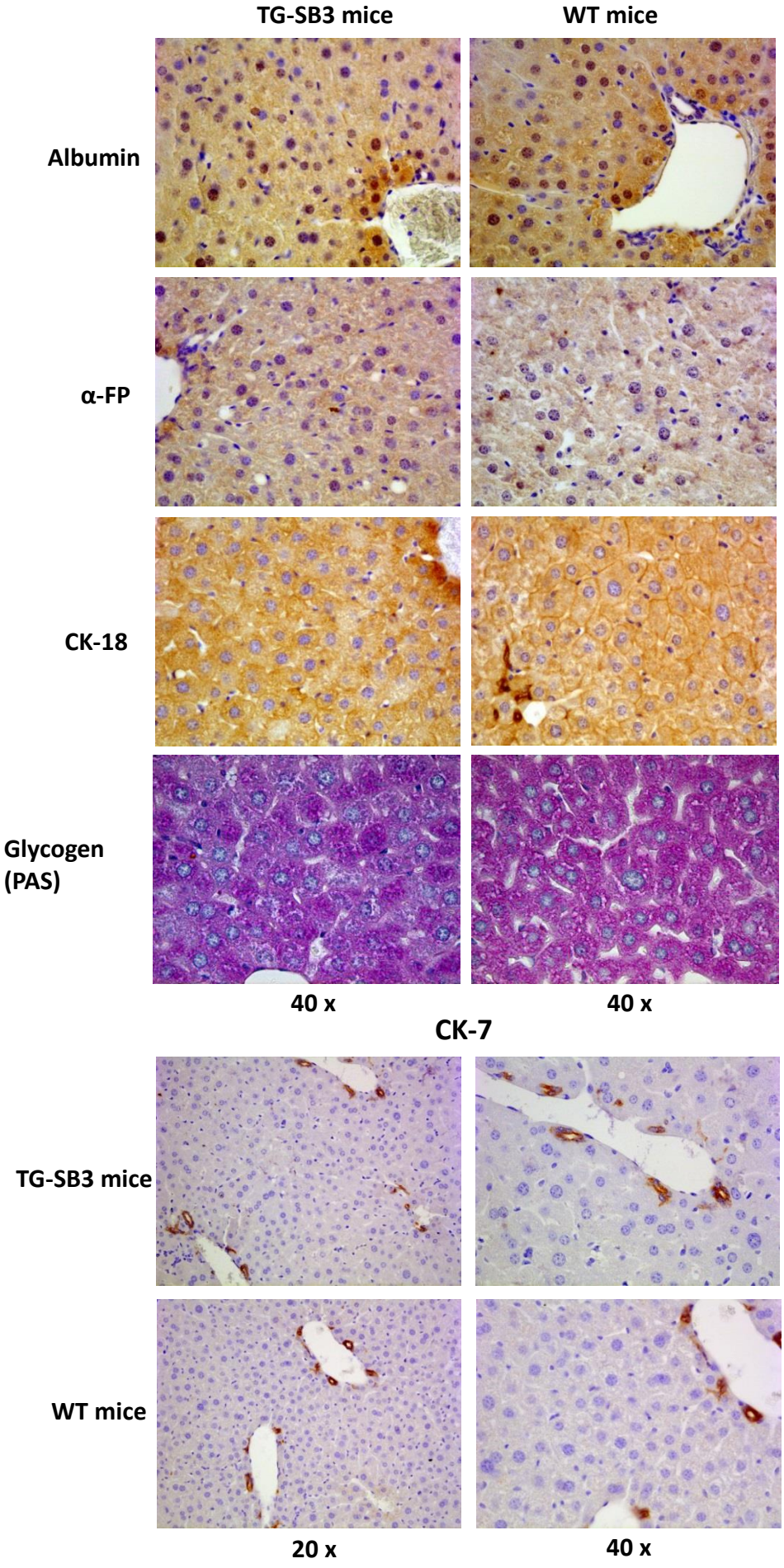
Supplementary Figure S 1.



Supplementary Figure S 2



Supplementary Figure S 3



GENERAL DISCUSSION

Available data in the scientific related literature have long been proposing a role of hypoxia and hypoxia inducible factors (HIFs) in the progression of CLD (Wilson et al., 2014). This occurs via promotion of several transcriptional programs like metabolic adaptation, angiogenesis, cell survival, epithelial mesenchymal transition (to name just a few). However, limited data are available for a condition of high clinical impact such as progressive NAFLD and the role of HIFs in the modulation of lipid metabolism. As previously mentioned, a study which employed mouse models with Cre-lox mediated deletion of VHL, HIF1α and/or HIF2α (Qu et al., 2011), suggested that HIF-2α, rather than HIF-1α, may play a major role in regulating hepatic lipid accumulation by various mechanisms, including up-regulation of lipid biosynthetic pathways and suppression of fatty acid-oxidation, then sustaining progression of this CLD. These considerations are consistent with what was observed in the first study of this thesis through immunohistochemistry analysis performed on liver biopsies from NAFLD/NASH patients with different stage of disease. In the majority of the liver sections a nuclear localization of HIF-2α is evident, indicating that can act as a transcription factor. Based on this data, the general aim of the first published paper was to evaluate the role of HIF-2α in the development of hepatic fibrosis by employing experimental models of progressive and dietary-induced progressive NAFLD/NASH. According to the predominant hepatocellular localization of HIF-2α observed in both human patients and mice fed with diets-induced steatohepatitis, we employed genetically manipulated mice carrying hepatocyte-specific deletion of HIF-2α that were then subjected to CDAA and MCD diets. Data presented in the first study reported in a mechanistical way that the hepatocyte-specific deletion of HIF-2α is able to alter and significantly decrease the progression of experimental dietary models of progressive NAFLD/NASH. This conclusion is based on the results obtained in mice carrying the conditional deletion of HIF-2α and fed with choline deficient-diet that, compared to control littermates, presented: i) a significant decrease of alanine aminotransferase (ALT) serum levels and hepatic TG; ii) the reduction in the number of α-SMA positive myofibroblasts and ECM deposition as well as the decrease of transcript levels of the main pro-fibrogenic markers. All together these results indicate that, in this model of experimental NAFLD, HIF-2α is relevant in order to determine hepatic steatosis and fibrosis. The data are in line with was previously obtained from other research groups

that used transgenic mice with conditional hepatocellular deletion of the gene encoding VHL (relevant for the degradation of HIF α-subunits) and combined with those of HIF-1α and/or HIF-2α but either without exposure to liver injury (Rankin et al., 2009) or following a short protocol (2 weeks) of ethanol exposure (Qu et al., 2011). Concerning NAFLD/NASH, in the experiments in which hepatocyte-specific deletion of VHL was combined with hepatocyte-specific deletion of HIF-1α, it has been observed the development of severe hepatic steatosis associated with impaired fatty acid β-oxidation, decreased lipogenic gene expression, and increased lipid storage capacity. By contrast, the Vhlh/Hif-2α mutant mice were phenotypically normal and appeared similar to wild-type animals. These findings demonstrated that HIF-2α, compared to HIF-1α, functions as an important regulator of hepatic lipid metabolism (Rankin et al., 2009). Indeed the data obtained in our study regarding the decrease of steatosis in mouse model with hepatocyte-specific deletion of HIF-2α are to be ascribed to the removal of the HIF-2α regulatory effects on lipid metabolism since real-time qPCR analysis also revealed a significant decrease in *sterol regulatory element binding factor 1* (Srebp1) and *fatty acid synthase* (Fasn). Previously published studies were however induced in a model of forced genetic manipulation and in the absence of hepatocellular chronic damage (Rankin et al., 2009) or in a model that did not result in hepatic fibrosis (2 weeks of ethanol administration; Qu et al., 2011). The fact that the experimental model of progressive NAFLD used in our study is similar to the corresponding human pathology reinforces our results. Our study is the first to report the specific HIF-2α overexpression in hepatocytes during the development of human NAFLD and to document the reduction of hepatic fibrosis.

On the basis of murine model employed in the first study (hepatocellular conditional deletion of HIF-2α) and the overall analysis of results obtained, we came to the conclusion that the reduction of hepatic fibrosis should be attributed to a lower release of one or more pro-fibrogenic mediators released by hepatocytes in a hypoxia/HIF-2α dependent manner. Along these lines, recent research has outlined the role of histidine-rich glycoprotein (HRGP), an abundant plasma protein synthesized by hepatocytes, which has a multifaceted role in the progression of human tumors other than HCC (Johnson et al., 2014). Moreover, this protein has been recently proposed to act as a hepatocyte-derived cytokine able to induce M1 macrophage differentiation, supporting inflammatory response in either experimental and clinical NAFLD/NASH as well as in chronic HCV and in the chronic CCl_4 murine model of CLD (Bartneck et al., 2016).

Accordingly, it is well known in literature that in the course of genesis and progression of the inflammatory response of CLD, activated liver macrophages play a significant role (Caligiuri et al., 2016). In particular, during NAFLD, Kuppfer cells are exposed to a variety of exogenous and endogenous stimuli, such as signals released by steatotic hepatocytes and toxic lipids that promote M1 macrophage polarization (Murray & Wynn, 2011); Kupffer cells are believed to be able, mainly through the secretion of CCL2 cytokine, to recruit the circulating monocytes. Moreover, during fibrogenesis, profibrotic macrophages produce various mediators, including TGFβ1, PDGF and insulin-like growth factor 1, that can directly activate fibroblasts/myofibroblasts which are responsible for excess ECM deposition. In addition, Kupffer cells and recruited monocyte/macrophages secrete various pro-fibrotic cytokines and chemokines, including IL-1β and TGFβ1 (Murray & Wynn, 2011).

Taken together, these data propmted us to investigate the pro-inflammatory role of HRGP in our experimental models of progressive NAFLD. The results obtained showed that the improvement of NAFLD evolution in HIF-2α deficient mice was associated with a selective lowering of the hepatic production of HRGP. Accordingly, qPCR, immunohistochemistry and hepatic leucocytes flow cytometry analyses showed a decrease of transcript levels of selected pro-inflammatory cytokines, a reduction in the number of $Ly6C^{high}/CD11b^{high}/F4/80^{high}$ inflammatory macrophages as well as a decreased intracellular expression of the M1 cytokine IL-12 in HIF-2α fl/fl/Alb-$Cre^{+/+}$ mice exposed to dietary models of NAFLD. Based on these results we hypothesized a potential interaction between HIF-2α and HRGP. Performing in vitro experiments, we observed that up-regulation of HIF-2α in HepG2 cells resulted in enhanced HRGP expression. Furthermore, the same cells silenced for HIF-2α and then exposed to hypoxic conditions showed a significant decrease also in HRGP transcript levels. Additionally, in liver biopsies from NAFLD patients HRGP immune-staining co-localizes with that of HIF-2α; moreover, in the same subjects a positive linear correlation was outlined between HIF-2α and HRGP transcript levels. These findings confirm that HRGP is mechanistically involved in the progression of fibrosis during experimental NAFLD by sustaining macrophage M1 polarization; moreover, our data indicate that HRGP production is strictly dependent on HIF-2α. This overall issue is in agreement with data obtained in the last years suggesting that hypoxia can actively affect inflammatory process (Lin & Simon, 2016; Taylor et al., 2016); these results can represent a potentially significant step forward in order to understand the regulation of oxygen-sensitive signaling pathways in immune cell subtypes that are resident within the inflamed tissue.

Concerning the hypoxia dependent-molecular factors involved in the progression of chronic inflammatory liver diseases, several studies have also outlined the role of Serpin B3 (SB3), a member of serine-proteases inhibitors (Biasiolo et al., 2008; Pontisso, 2009, 2014; Turato et al., 2010). A close relationship between Serpin B3 and hypoxia has recently been outlined in our laboratory in a study showing that SB3 in HCC cell lines is up-regulated through a HIF-2α-dependent and ROS-modulated mechanism (Cannito et al., 2015). Moreover, although in normal human livers SB3 expression is virtually undetectable, several reports have shown the presence of Serpin B3 in liver biopsies from patients with CLD (etiology chronic HCV infection), and SerpinB3 expression has been associated to CLD progression and liver carcinogenesis (Biasiolo et al., 2008; Turato et al., 2014). Indeed, in CLD patients Serpin B3 up-regulation correlates with TGFβ1 expression and the extent of hepatic fibrosis (Turato et al., 2010). Furthermore, experiments using hepatocyte-derived cell lines over-expressing Serpin B3 have evidenced its role in promoting TGFβ1 production. Based on these considerations, the second study included in the present thesis (published paper n.2) was designed to verify the hypothesis that Serpin B3 can act as a pro-fibrogenic mediator during the progression of CLD. The first series of experiments presented in the study was planned to analyze the biological action of SB3 released by hepatocytes and, possibly, also by activated MFs (as immunohistochemistry analysis in human cirrhotic livers seems to suggest). In particular, we investigated the putative ability of this protein to exert pro-fibrogenic effects on the surrounding cell populations, in particular MFs. The results obtained in these experiments indicate the following: i) the human recombinant SB3 can directly up-regulate the expression of key-profibrogenic and proangiogenic genes in LX2 cells as well as in primary cultures of human HSC/MFs; these data are of particular relevance since this protein may combine pro-fibrogenic and pro-angiogenic activities. ii) the human recombinant SB3 is unable to directly evoke a proliferative response in HSC/MFs; iii) the human recombinant SB3 is instead able to significantly stimulate the oriented migration of HSC/MFs (assays in Boyden chambers) as it happens in the presence of other established chemotactic polypeptides such as, in particular, the PDGF-BB, which is used as a reference chemiotactic polypeptide for these cells. In relation to the pro-migratory effect exerted by SB3, the data here presented are fully compatible with those already published by our laboratory (Novo et al., 2011; 2012; Busletta et al., 2011) for both HSC cells/MFs and mesenchymal stem cells, obtained from hemopoietic bone marrow exhibiting a fibroblastoid phenotype, when exposed to different chemotactic polypeptides. SB3, like

other chemotactic polypeptides such as PDGF-BB, VEGF-A and MCP-1, induced oriented migration, following ligand-receptor interaction, that involved an early and transient increase of the intracellular levels of ROS, likely due to parallel involvement of the plasma-membrane NADPH-oxidase, and subsequent activation of terminal c-Jun-NH2 kinase isoforms (JNK1/2).

The data obtained in these analyses, potentially compatible with the hypothesis of a pro-fibrogenic role of SB3, have suggested to extend the study to two *in vivo* experimental murine models of hepatic fibrosis: i) the chronic administration of CCl_4, resembling pan-lobular/parenchymal fibrosis found in the clinical conditions of cirrhosis and HCC; ii) the methionine and choline-deficient diet (MCD) that leads to pericellular/perisinusoidal fibrosis similar to that seen in human NAFLD/NASH. The two protocols for experimental fibrosis were performed in C57 Bl6 wild type mice and transgenic mice of the same mouse strain genetically manipulated in order to overexpress SB3 in the hepatic parenchyma.

Hepatocellular overexpression of SB3 in transgenic mice resulted in a significant increase in the deposition of collagen fibers associated with a significant increase in the number of α-SMA-positive myofibroblasts compared to what was observed in wild t*ype* mice exposed to both liver injury models, confirming the experimental hypothesis. The pro-fibrogenic action of SB3 was characterized by increased mRNA levels of a set of genes encoding proteins that are notoriously overexpressed in conditions of fibrosis. The increased transcription of collagen type I, TIMP-1 and TGFβ1 represents a real pro-fibrogenic *signature* (Iredale, 2007; Parola et al., 2008; Povero et al., 2010), which is compatible with the morphological data observed in transgenic mice versus the wild type mice, then suggesting the existence of a direct causal link between hepatic overexpression of SB3 and the pro-fibrogenic behavior of hepatic MFs. This statement is based on the following experimental evidence: i) genetic manipulation of these transgenic mice is such that overexpress SB3 only in hepatocytes (Villano et al., 2010) and not in other hepatic cell populations; ii) Stable transfected HepG2 cells in order to over-express the SB3 progressively release the protein in the culture medium (Quarta et al., 2010) and it is therefore likely that this occurs also in vivo for hepatocytes of transgenic mice; iii) the transcripts of collagen type I and TIMP-1 are known to be primarily produced by HSC/MFs and, more generally, by hepatic MFs in the course of fibrogenic progression of CLD.

Thus, in conclusion Serpin B3 production by hepatocytes (and perhaps by HSC/MFs) in the liver is modulated by hypoxia through HIF2α-dependent mechanisms and can contribute to the fibrogenic progression of CLDs both indirectly, stimulating TGF-β1

synthesis in surrounding HSC/MFs, then directly favoring the recruitment/migration of MFs cells. Moreover, S-B3 may also promote fibrogenic progression by stimulating the proliferation of hepatic MFs as a consequence of the increased transcription of PDGF-B (the most potent mitogenic agent for the HSC/MFs), even if remains to be established which cells respond in this way to the action of SB3 itself.

Finally, the studies presented in this thesis overall demonstrates that HIF-2α-dependent mediators released by hepatocytes during CLDs, like HRGP and SB3, are mechanistically involved in the progression of experimental hepatic fibrosis. These findings may help to elucidate the mechanism(s) involved in NAFLD progression and propose HIF-2α and HIF-2α-dependent genes as putative targets for future therapeutic strategies in NAFLD since, at present, we lack biomarkers to predict the individual risk of progression and HCC development as well as validated therapeutic approaches for a disease affecting a high percentage of individuals in the general population.

REFERENCES

Albano, E. (2008). Oxidative mechanisms in the pathogenesis of alcoholic liver disease. *Molecular Aspects of Medicine*, *29*(1–2), 9–16. https://doi.org/10.1016/j.mam.2007.09.004

Angulo, P. (2002). Nonalcoholic Fatty Liver Disease. *New England Journal of Medicine*, *346*(16), 1221–1231. https://doi.org/10.1056/NEJMra011775

Argo, C. K., & Caldwell, S. H. (2009). Epidemiology and natural history of non-alcoholic steatohepatitis. *Clinics in Liver Disease*, *13*(4), 511–31. https://doi.org/10.1016/j.cld.2009.07.005

Arora, A., & Sharma, P. (2012). Non-invasive Diagnosis of Fibrosis in Non-alcoholic Fatty Liver Disease. *Journal of Clinical and Experimental Hepatology*, *2*(2), 145–155. https://doi.org/10.1016/S0973-6883(12)60103-0

Arun, J., Clements, R., Lazenby, A., Leeth, R., & Abrams, G. (2006). The Prevalence of Nonalcoholic Steatohepatitis is Greater in Morbidly Obese Men Compared to Women. *Obesity Surgery*, *16*(10), 1351–1358. https://doi.org/10.1381/096089206778663715

Bangoura, G., Liu, Z.-S., Qian, Q., Jiang, C.-Q., Yang, G.-F., & Jing, S. (2007). Prognostic significance of HIF-2alpha/EPAS1 expression in hepatocellular carcinoma. *World Journal of Gastroenterology*, *13*(23), 3176–82. https://doi.org/10.3748/WJG.V13.I23.3176

Bartneck, M., Fech, V., Ehling, J., Govaere, O., Warzecha, K. T., Hittatiya, K., … Tacke, F. (2016). Histidine-rich glycoprotein promotes macrophage activation and inflammation in chronic liver disease. *Hepatology*, *63*(4), 1310–1324. https://doi.org/10.1002/hep.28418

Bertout, J. A., Patel, S. A., & Simon, M. C. (2008). The impact of O2 availability on human cancer. *Nature Reviews. Cancer*, *8*(12), 967–75. https://doi.org/10.1038/nrc2540

Biasiolo, A., Chemello, L., Quarta, S., Cavalletto, L., Bortolotti, F., Caberlotto, C., … Pontisso, P. (2008). Monitoring SCCA-IgM complexes in serum predicts liver disease progression in patients with chronic hepatitis. *Journal of Viral Hepatitis*, *15*(4), 246–249. https://doi.org/10.1111/j.1365-2893.2007.00935.x

Brahimi-Horn, M. C., & Pouysségur, J. (2007). Oxygen, a source of life and stress. *FEBS Letters*, *581*(19), 3582–3591. https://doi.org/10.1016/j.febslet.2007.06.018

Browning, J. D., & Horton, J. D. (2004). Molecular mediators of hepatic steatosis and liver injury. *The Journal of Clinical Investigation*, *114*(2), 147–52. https://doi.org/10.1172/JCI22422

Busletta, C., Novo, E., Valfrè Di Bonzo, L., Povero, D., Paternostro, C., Ievolella, M., … Parola, M. (2011). Dissection of the Biphasic Nature of Hypoxia-Induced Motogenic Action in Bone Marrow-Derived Human Mesenchymal Stem Cells. *STEM CELLS*, *29*(6), 952–963. https://doi.org/10.1002/stem.642

Calabrese, F., Lunardi, F., Giacometti, C., Marulli, G., Gnoato, M., Pontisso, P., … Agostini, C. (2008). Overexpression of squamous cell carcinoma antigen in idiopathic pulmonary fibrosis: clinicopathological correlations. *Thorax*, *63*(9), 795–802. https://doi.org/10.1136/thx.2007.088583

Caligiuri, A., Gentilini, A., & Marra, F. (2016). Molecular Pathogenesis of NASH. *International Journal of Molecular Sciences*, *17*(10), 1575. https://doi.org/10.3390/ijms17091575

Cannito, S., Morello, E., Bocca, C., Foglia, B., Benetti, E., Novo, E., … Parola, M. (2017). Microvesicles released from fat-laden cells promote activation of hepatocellular NLRP3 inflammasome: A pro-inflammatory link between lipotoxicity and non-alcoholic

steatohepatitis. *PLOS ONE*, *12*(3), e0172575. https://doi.org/10.1371/journal.pone.0172575

Cannito, S., Novo, E., Compagnone, A., Valfrè di Bonzo, L., Busletta, C., Zamara, E., … Parola, M. (2008). Redox mechanisms switch on hypoxia-dependent epithelial–mesenchymal transition in cancer cells. *Carcinogenesis*, *29*(12), 2267–2278. https://doi.org/10.1093/carcin/bgn216

Cannito, S., Novo, E., & Parola, M. (2017). Therapeutic pro-fibrogenic signaling pathways in fibroblasts. *Advanced Drug Delivery Reviews*. https://doi.org/10.1016/j.addr.2017.05.017

Cannito, S., Paternostro, C., Busletta, C., Bocca, C., Colombatto, S., Miglietta, A., … Parola, M. (2014). Hypoxia, hypoxia-inducible factors and fibrogenesis in chronic liver diseases. *Histology and Histopathology*. https://doi.org/10.14670/HH-29.33

Cannito, S., Turato, C., Paternostro, C., Biasiolo, A., Colombatto, S., Cambieri, I., … Parola, M. (2015, February 10). Hypoxia up-regulates SERPINB3 through HIF-2α in human liver cancer cells. *Oncotarget*. Impact Journals. https://doi.org/10.18632/oncotarget.2943

Chan, D. A., Kawahara, T. L. A., Sutphin, P. D., Chang, H. Y., Chi, J.-T., & Giaccia, A. J. (2009). Tumor vasculature is regulated by PHD2-mediated angiogenesis and bone marrow-derived cell recruitment. *Cancer Cell*, *15*(6), 527–38. https://doi.org/10.1016/j.ccr.2009.04.010

Chaurasia, B., & Summers, S. A. (2015). Ceramides – Lipotoxic Inducers of Metabolic Disorders. *Trends in Endocrinology & Metabolism*, *26*(10), 538–550. https://doi.org/10.1016/j.tem.2015.07.006

Cobbina, E., & Akhlaghi, F. (2017). Non-alcoholic fatty liver disease (NAFLD) – pathogenesis, classification, and effect on drug metabolizing enzymes and transporters. *Drug Metabolism Reviews*, *49*(2), 197–211. https://doi.org/10.1080/03602532.2017.1293683

Copple, B. L. (2010). Hypoxia stimulates hepatocyte epithelial to mesenchymal transition by hypoxia-inducible factor and transforming growth factor-β-dependent mechanisms. *Liver International*, *30*(5), 669–682. https://doi.org/10.1111/j.1478-3231.2010.02205.x

Copple, B. L., Bai, S., Burgoon, L. D., & Moon, J.-O. (2011). Hypoxia-inducible factor-1α regulates the expression of genes in hypoxic hepatic stellate cells important for collagen deposition and angiogenesis. *Liver International*, *31*(2), 230–244. https://doi.org/10.1111/j.1478-3231.2010.02347.x

Copple, B. L., Bai, S., & Moon, J.-O. (2010). Hypoxia-inducible factor-dependent production of profibrotic mediators by hypoxic Kupffer cells. *Hepatology Research : The Official Journal of the Japan Society of Hepatology*, *40*(5), 530–9. https://doi.org/10.1111/j.1872-034X.2010.00635.x

Copple, B. L., Bustamante, J. J., Welch, T. P., Kim, N. D., & Moon, J.-O. (2009). Hypoxia-inducible factor-dependent production of profibrotic mediators by hypoxic hepatocytes. *Liver International*, *29*(7), 1010–1021. https://doi.org/10.1111/j.1478-3231.2009.02015.x

Copple, B. L., Kaska, S., & Wentling, C. (2012). Hypoxia-inducible factor activation in myeloid cells contributes to the development of liver fibrosis in cholestatic mice. *The Journal of Pharmacology and Experimental Therapeutics*, *341*(2), 307–16. https://doi.org/10.1124/jpet.111.189340

Corpechot, C., Barbu, V., Wendum, D., Chignard, N., Housset, C., Poupon, R., & Rosmorduc, O. (2002). Hepatocyte Growth Factor and c-Met Inhibition by Hepatic Cell Hypoxia. *The American Journal of Pathology*, *160*(2), 613–620. https://doi.org/10.1016/S0002-9440(10)64881-X

Corpechot, C., Barbu, V., Wendum, D., Kinnman, N., Rey, C., Poupon, R., … Rosmorduc, O. (2002). Hypoxia-induced VEGF and collagen I expressions are associated with angiogenesis

and fibrogenesis in experimental cirrhosis. *Hepatology (Baltimore, Md.)*, *35*(5), 1010–21. https://doi.org/10.1053/jhep.2002.32524

De Minicis, S., Agostinelli, L., Rychlicki, C., Sorice, G. P., Saccomanno, S., Candelaresi, C., … Svegliati-Baroni, G. (2014). HCC development is associated to peripheral insulin resistance in a mouse model of NASH. *PloS One*, *9*(5), e97136. https://doi.org/10.1371/journal.pone.0097136

De Minicis, S., & Brenner, D. A. (2007). NOX in liver fibrosis. *Archives of Biochemistry and Biophysics*, *462*(2), 266–72. https://doi.org/10.1016/j.abb.2007.04.016

De Minicis, S., Rychlicki, C., Agostinelli, L., Saccomanno, S., Candelaresi, C., Trozzi, L., … Svegliati-Baroni, G. (2014). Dysbiosis contributes to fibrogenesis in the course of chronic liver injury in mice. *Hepatology*, *59*(5), 1738–1749. https://doi.org/10.1002/hep.26695

Dengler, V. L., Galbraith, M. D., & Espinosa, J. M. (2014). Critical Reviews in Biochemistry and Molecular Biology Transcriptional regulation by hypoxia inducible factors Transcriptional regulation by hypoxia inducible factors. *Critical Reviews in Biochemistry and Molecular Biology Cox Crit Rev Biochem Mol Biol*, *49*(491), 1–15. https://doi.org/10.3109/10409238.2013.838205

Elvert, G., Kappel, A., Heidenreich, R., Englmeier, U., Lanz, S., Acker, T., … Flamme, I. (2003). Cooperative interaction of hypoxia-inducible factor-2α (HIF-2α) and Ets-1 in the transcriptional activation of vascular endothelial growth factor receptor-2 (Flk-1). *Journal of Biological Chemistry*, *278*(9), 7520–7530. https://doi.org/10.1074/jbc.M211298200

Erler, J. T., Bennewith, K. L., Cox, T. R., Lang, G., Bird, D., Koong, A., … Giaccia, A. J. (2009). Hypoxia-Induced Lysyl Oxidase Is a Critical Mediator of Bone Marrow Cell Recruitment to Form the Premetastatic Niche. *Cancer Cell*, *15*(1), 35–44. https://doi.org/10.1016/j.ccr.2008.11.012

Flegal, K. M., Kruszon-Moran, D., Carroll, M. D., Fryar, C. D., & Ogden, C. L. (2016). Trends in Obesity Among Adults in the United States, 2005 to 2014. *JAMA*, *315*(21), 2284. https://doi.org/10.1001/jama.2016.6458

Fraisl, P., Mazzone, M., Schmidt, T., & Carmeliet, P. (2009). Regulation of Angiogenesis by Oxygen and Metabolism. *Developmental Cell*, *16*(2), 167–179. https://doi.org/10.1016/j.devcel.2009.01.003

Franovic, A., Holterman, C. E., Payette, J., & Lee, S. (2009). Human cancers converge at the HIF-2α oncogenic axis. *Proceedings of the National Academy of Sciences*, *106*(50), 21306–21311. https://doi.org/10.1073/pnas.0906432106

Furuta, E., Pai, S. K., Zhan, R., Bandyopadhyay, S., Watabe, M., Mo, Y.-Y., … Watabe, K. (2008). Fatty acid synthase gene is up-regulated by hypoxia via activation of Akt and sterol regulatory element binding protein-1. *Cancer Research*, *68*(4), 1003–11. https://doi.org/10.1158/0008-5472.CAN-07-2489

Gardner, H., Broberg, A., Pozzi, A., Laato, M., & Heino, J. (1999). Absence of integrin alpha1beta1 in the mouse causes loss of feedback regulation of collagen synthesis in normal and wounded dermis. *Journal of Cell Science*, *112 (Pt 3)*, 263–72. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/9885280

Gettins, P. G. W. (2002). Serpin structure, mechanism, and function. *Chemical Reviews*, *102*(12), 4751–804. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/12475206

Gilany, K., & Vafakhah, M. (2010). Hypoxia : a Review. *Journal of Paramedical Sciences*, *1*(2), 43–60.

Gram, J., Jespersen, J., Ingeberg, S., Bentsen, K. D., & Bach, E. (1985). Plasma histidine-rich

glycoprotein and plasminogen in patients with liver disease. *Thrombosis Research*, *39*(4), 411–7. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/4049324

Guido, M., Roskams, T., Pontisso, P., Fassan, M., Thung, S. N., Giacomelli, L., … Rugge, M. (2008). Squamous cell carcinoma antigen in human liver carcinogenesis. *Journal of Clinical Pathology*, *61*(4), 445–7. https://doi.org/10.1136/jcp.2007.051383

Guzy, R. D., & Schumacker, P. T. (2006). Oxygen sensing by mitochondria at complex III: the paradox of increased reactive oxygen species during hypoxia. *Experimental Physiology*, *91*(5), 807–19. https://doi.org/10.1113/expphysiol.2006.033506

Hansen, H. H., Feigh, M., Veidal, S. S., Rigbolt, K. T., Vrang, N., & Fosgerau, K. (2017). Mouse models of nonalcoholic steatohepatitis in preclinical drug development. *Drug Discovery Today*, *0*(0). https://doi.org/10.1016/j.drudis.2017.06.007

Hoshida, Y., Toffanin, S., Lachenmayer, A., Villanueva, A., Minguez, B., & Llovet, J. M. (2010). Molecular classification and novel targets in hepatocellular carcinoma: recent advancements. *Seminars in Liver Disease*, *30*(1), 35–51. https://doi.org/10.1055/s-0030-1247131

Huang, B., Wu, P., Bowker-Kinley, M. M., & Harris, R. A. (2002). Regulation of pyruvate dehydrogenase kinase expression by peroxisome proliferator-activated receptor-alpha ligands, glucocorticoids, and insulin. *Diabetes*, *51*(2), 276–83. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11812733

Ibrahim, J., Nguyen, A. H., Rehman, A., Ochi, A., Jamal, M., Graffeo, C. S., … Miller, G. (2012). Dendritic Cell Populations With Different Concentrations of Lipid Regulate Tolerance and Immunity in Mouse and Human Liver. *Gastroenterology*, *143*(4), 1061–1072. https://doi.org/10.1053/j.gastro.2012.06.003

Imtiyaz, H. Z., & Simon, M. C. (2010). Hypoxia-Inducible Factors as Essential Regulators of Inflammation. In *Current topics in microbiology and immunology* (Vol. 345, pp. 105–120). https://doi.org/10.1007/82_2010_74

Iredale, J. P. (2007). Models of liver fibrosis: exploring the dynamic nature of inflammation and repair in a solid organ. *Journal of Clinical Investigation*, *117*(3), 539–548. https://doi.org/10.1172/JCI30542

Johnson, L. D. S., Goubran, H. A., & Kotb, R. R. (2014). Histidine rich glycoprotein and cancer: a multi-faceted relationship. *Anticancer Research*, *34*(2), 593–603. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/24510988

Jones, A. L., Hulett, M. D., & Parish, C. R. (2005). Histidine-rich glycoprotein: A novel adaptor protein in plasma that modulates the immune, vascular and coagulation systems. *Immunology and Cell Biology*, *83*(2), 106–18. https://doi.org/10.1111/j.1440-1711.2005.01320.x

Ju, C., Colgan, S. P., & Eltzschig, H. K. (2016). Hypoxia-inducible factors as molecular targets for liver diseases. *J Mol Med*, *94*, 613–627. https://doi.org/10.1007/s00109-016-1408-1

Jungermann, K., & Katz, N. (1989). Functional specialization of different hepatocyte populations. Retrieved from https://www.scopus.com/record/display.uri?eid=2-s2.0-0024336445&origin=inward&txGid=ecf1b62196a43aa8f13fa148a100d943

Kaelin Jr, W. G. (2008). The von Hippel–Lindau tumour suppressor protein: O2 sensing and cancer. *Nature Reviews Cancer*, *8*(11), 865–873. https://doi.org/10.1038/nrc2502

Kapoor, A., & Sanyal, A. J. (2009). Endoplasmic Reticulum Stress and the Unfolded Protein Response. *Clinics in Liver Disease*, *13*(4), 581–590. https://doi.org/10.1016/j.cld.2009.07.004

Katz, N., & Jungermann, K. (1976). Autoregulatory shift from fructolysis to lactate gluconeogenisis in rat hepatocyte suspensions. The problem of metabolic zonation of liver

parenchyma. *Hoppe-Seyler's Zeitschrift Fur Physiologische Chemie*, *357*(3), 359–75. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/955564

Keith, B., Johnson, R. S., & Simon, M. C. (2011). HIF1α and HIF2α: sibling rivalry in hypoxic tumour growth and progression. *Nature Reviews Cancer*, *12*(1), 9–22. https://doi.org/10.1038/nrc3183

Khan, M. S., Singh, P., Azhar, A., Naseem, A., Rashid, Q., Kabir, M. A., & Jairajpuri, M. A. (2011). Serpin Inhibition Mechanism: A Delicate Balance between Native Metastable State and Polymerization. *Journal of Amino Acids*, *2011*, 1–10. https://doi.org/10.4061/2011/606797

Kietzmann, T. (2017). Metabolic zonation of the liver: The oxygen gradient revisited. *Redox Biology*. https://doi.org/10.1016/j.redox.2017.01.012

Kim, J.-W. (2010). Hypoxia-inducible factor 1, hepatocellular carcinoma and angiogenesis. *The Korean Journal of Hepatology*, *16*(3), 278–9. https://doi.org/10.3350/kjhep.2010.16.3.278

Kinnman, N., & Housset, C. (2002). Peribiliary myofibroblasts in biliary type liver fibrosis. *Frontiers in Bioscience : A Journal and Virtual Library*, *7*, d496-503. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11815289

Kleiner, D. E., Brunt, E. M., Van Natta, M., Behling, C., Contos, M. J., Cummings, O. W., … Nonalcoholic Steatohepatitis Clinical Research Network. (2005). Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology*, *41*(6), 1313–1321. https://doi.org/10.1002/hep.20701

Koury, S. T., Bondurant, M. C., Koury, M. J., & Semenza, G. L. (1991). Localization of cells producing erythropoietin in murine liver by in situ hybridization, *77*(11), 2497–2503. Retrieved from https://www.scopus.com/record/display.uri?eid=2-s2.0-0025912710&origin=inward&txGid=762190bacf3aabfd92ef30e3c291d8d8

Kubes, P., & Mehal, W. Z. (2012). Sterile Inflammation in the Liver. *Gastroenterology*, *143*(5), 1158–1172. https://doi.org/10.1053/j.gastro.2012.09.008

Lackner, C., Gogg-Kamerer, M., Zatloukal, K., Stumptner, C., Brunt, E. M., & Denk, H. (2008). Ballooned hepatocytes in steatohepatitis: The value of keratin immunohistochemistry for diagnosis. *Journal of Hepatology*, *48*(5), 821–828. https://doi.org/10.1016/j.jhep.2008.01.026

Leamy, A. K., Egnatchik, R. A., & Young, J. D. (2013). Molecular mechanisms and the role of saturated fatty acids in the progression of non-alcoholic fatty liver disease. *Progress in Lipid Research*, *52*(1), 165–174. https://doi.org/10.1016/j.plipres.2012.10.004

Lemoinne, S., Cadoret, A., El Mourabit, H., Thabut, D., & Housset, C. (2013). Origins and functions of liver myofibroblasts. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1832*(7), 948–954. https://doi.org/10.1016/j.bbadis.2013.02.019

Li, C., Zhang, Z., Zhang, P., & Liu, J. (2014). Diagnostic accuracy of des-gamma-carboxy prothrombin versus α-fetoprotein for hepatocellular carcinoma: A systematic review. *Hepatology Research : The Official Journal of the Japan Society of Hepatology*, *44*(10), E11-25. https://doi.org/10.1111/hepr.12201

Li, S., Yao, D., Wang, L., Wu, W., Qiu, L., Yao, M., … Ni, Q. (2011). Expression characteristics of hypoxia-inducible factor-1α and its clinical values in diagnosis and prognosis of hepatocellular carcinoma. *Hepatitis Monthly*, *11*(10), 821–8. https://doi.org/10.5812/kowsar.1735143X.771

Liedtke, C., Luedde, T., Sauerbruch, T., Scholten, D., Streetz, K., Tacke, F., … Weiskirchen, R. (2013). Experimental liver fibrosis research: update on animal models, legal issues and translational aspects. *Fibrogenesis & Tissue Repair*, *6*(1), 19. https://doi.org/10.1186/1755-

1536-6-19

Lin, N., & Simon, M. C. (2016a). Hypoxia-inducible factors: key regulators of myeloid cells during inflammation. *Journal of Clinical Investigation*, *126*(10), 3661–3671. https://doi.org/10.1172/JCI84426

Lin, N., & Simon, M. C. (2016b). Hypoxia-inducible factors: key regulators of myeloid cells during inflammation. *The Journal of Clinical Investigation*, *126*(10), 3661–3671. https://doi.org/10.1172/JCI84426

Lonardo, A., Nascimbeni, F., Maurantonio, M., Marrazzo, A., Rinaldi, L., & Adinolfi, L. E. (2017). Nonalcoholic fatty liver disease: Evolving paradigms. *World Journal of Gastroenterology*, *23*(36), 6571–6592. https://doi.org/10.3748/wjg.v23.i36.6571

Lonardo, A., Nascimbeni, F., Targher, G., Bernardi, M., Bonino, F., Bugianesi, E., … Bellentani, S. (2017). AISF position paper on nonalcoholic fatty liver disease (NAFLD): Updates and future directions. *Digestive and Liver Disease*, *49*(5), 471–483. https://doi.org/10.1016/j.dld.2017.01.147

Lotowska, J. M., Sobaniec-Lotowska, M. E., Bockowska, S. B., & Lebensztejn, D. M. (2014). Pediatric non-alcoholic steatohepatitis: the first report on the ultrastructure of hepatocyte mitochondria. *World Journal of Gastroenterology*, *20*(15), 4335–40. https://doi.org/10.3748/wjg.v20.i15.4335

Luo, D., Wang, Z., Wu, J., Jiang, C., & Wu, J. (2014). The role of hypoxia inducible factor-1 in hepatocellular carcinoma. *BioMed Research International*, *2014*, 409272. https://doi.org/10.1155/2014/409272

Majmundar, A. J., Wong, W. J., & Simon, M. C. (2010). Hypoxia-inducible factors and the response to hypoxic stress. *Mol Cell*, *40*(2), 294–309. https://doi.org/S1097-2765(10)00750-1 [pii]\r10.1016/j.molcel.2010.09.022

Mak, P., Leav, I., Pursell, B., Bae, D., Yang, X., Taglienti, C. A., … Mercurio, A. M. (2010). ERβ Impedes Prostate Cancer EMT by Destabilizing HIF-1α and Inhibiting VEGF-Mediated Snail Nuclear Localization: Implications for Gleason Grading. *Cancer Cell*, *17*(4), 319–332. https://doi.org/10.1016/j.ccr.2010.02.030

Marchesini, G., Brizi, M., Bianchi, G., Tomassetti, S., Bugianesi, E., Lenzi, M., … Melchionda, N. (2001). Nonalcoholic fatty liver disease: a feature of the metabolic syndrome. *Diabetes*, *50*(8), 1844–50. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/11473047

Marchesini, G., Brizi, M., Morselli-Labate, A. M., Bianchi, G., Bugianesi, E., McCullough, A. J., … Melchionda, N. (1999). Association of nonalcoholic fatty liver disease with insulin resistance. *The American Journal of Medicine*, *107*(5), 450–5. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10569299

Marra, F., & Bertolani, C. (2009). Adipokines in liver diseases. *Hepatology*, *50*(3), 957–969. https://doi.org/10.1002/hep.23046

Marra, F., & Tacke, F. (2014). Roles for chemokines in liver disease. *Gastroenterology*, *147*(3), 577–594.e1. https://doi.org/10.1053/j.gastro.2014.06.043

Martini, A., Gallotta, A., Pontisso, P., & Fassina, G. (2015, December 18). Clinical applications of squamous cell carcinoma antigen-immunoglobulins M to monitor chronic hepatitis C. *World Journal of Hepatology*. Baishideng Publishing Group Inc. https://doi.org/10.4254/wjh.v7.i29.2913

McClain, C. J., Barve, S., & Deaciuc, I. (2007). Good fat/bad fat. *Hepatology*, *45*(6), 1343–1346. https://doi.org/10.1002/hep.21788

Mederacke, I., Hsu, C. C., Troeger, J. S., Huebener, P., Mu, X., Dapito, D. H., … Schwabe, R. F. (2013). Fate tracing reveals hepatic stellate cells as dominant contributors to liver fibrosis independent of its aetiology. *Nature Communications*, *4*, 2823. https://doi.org/10.1038/ncomms3823

Mee, C. J., Harris, H. J., Farquhar, M. J., Wilson, G., Reynolds, G., Davis, C., … McKeating, J. A. (2009). Polarization restricts hepatitis C virus entry into HepG2 hepatoma cells. *Journal of Virology*, *83*(12), 6211–21. https://doi.org/10.1128/JVI.00246-09

Minamishima, Y. A., Moslehi, J., Padera, R. F., Bronson, R. T., Liao, R., & Kaelin, W. G. (2009). A feedback loop involving the Phd3 prolyl hydroxylase tunes the mammalian hypoxic response in vivo. *Molecular and Cellular Biology*, *29*(21), 5729–41. https://doi.org/10.1128/MCB.00331-09

Moon, E.-J., Jeong, C.-H., Jeong, J.-W., Kim, K. R., Yu, D.-Y., Murakami, S., … Kim, K.-W. (2003). Hepatitis B virus X protein induces angiogenesis by stabilizing hypoxia-inducible factor-1. *The FASEB Journal*, *18*(2), 382–4. https://doi.org/10.1096/fj.03-0153fje

Moon, J.-O., Welch, T. P., Gonzalez, F. J., & Copple, B. L. (2009). Reduced liver fibrosis in hypoxia-inducible factor-1alpha-deficient mice. *American Journal of Physiology. Gastrointestinal and Liver Physiology*, *296*(3), G582-92. https://doi.org/10.1152/ajpgi.90368.2008

Murray, P. J., & Wynn, T. A. (2011). Protective and pathogenic functions of macrophage subsets. *Nature Reviews. Immunology*, *11*(11), 723–37. https://doi.org/10.1038/nri3073

Nakamura, K., Zen, Y., Sato, Y., Kozaka, K., Matsui, O., Harada, K., & Nakanuma, Y. (2007). Vascular endothelial growth factor, its receptor Flk-1, and hypoxia inducible factor-1α are involved in malignant transformation in dysplastic nodules of the liver. *Human Pathology*, *38*(10), 1532–1546. https://doi.org/10.1016/J.HUMPATH.2007.03.002

Nakamura, S., Nouso, K., Sakaguchi, K., Ito, Y. M., Ohashi, Y., Kobayashi, Y., … Shiratori, Y. (2006). Sensitivity and Specificity of Des-Gamma-Carboxy Prothrombin for Diagnosis of Patients with Hepatocellular Carcinomas Varies According to Tumor Size. *The American Journal of Gastroenterology*, *101*(9), 2038–2043. https://doi.org/10.1111/j.1572-0241.2006.00681.x

Nasimuzzaman, M., Waris, G., Mikolon, D., Stupack, D. G., & Siddiqui, A. (2007). Hepatitis C virus stabilizes hypoxia-inducible factor 1alpha and stimulates the synthesis of vascular endothelial growth factor. *Journal of Virology*, *81*(19), 10249–57. https://doi.org/10.1128/JVI.00763-07

Nath, B., & Szabo, G. (2012). Hypoxia and hypoxia inducible factors: Diverse roles in liver diseases. *Hepatology*, *55*(2), 622–633. https://doi.org/10.1002/hep.25497

Neuschwander-Tetri, B. A. (2010). Hepatic lipotoxicity and the pathogenesis of nonalcoholic steatohepatitis: the central role of nontriglyceride fatty acid metabolites. *Hepatology (Baltimore, Md.)*, *52*(2), 774–88. https://doi.org/10.1002/hep.23719

Novo, E. (2012). The biphasic nature of hypoxia-induced directional migration of activated human hepatic stellate cells. *JOURNAL OF PATHOLOGY*, *226*, 588–597.

Novo, E., Busletta, C., Bonzo, L. V. di, Povero, D., Paternostro, C., Mareschi, K., … Parola, M. (2011). Intracellular reactive oxygen species are required for directional migration of resident and bone marrow-derived hepatic pro-fibrogenic cells. *Journal of Hepatology*, *54*(5), 964–974. https://doi.org/10.1016/j.jhep.2010.09.022

Novo, E., Cannito, S., Morello, E., Paternostro, C., Bocca, C., Miglietta, A., & Parola, M. (2015). Hepatic myofibroblasts and fibrogenic progression of chronic liver diseases. *Histology and Histopathology*, *30*(9), 1011–32. https://doi.org/10.14670/HH-11-623

Novo, E., Cannito, S., Paternostro, C., Bocca, C., Miglietta, A., & Parola, M. (2014). Cellular and molecular mechanisms in liver fibrogenesis. *Archives of Biochemistry and Biophysics*, *548*, 20–37. https://doi.org/10.1016/j.abb.2014.02.015

Novo, E., Marra, F., Zamara, E., Valfrè di Bonzo, L., Caligiuri, A., Cannito, S., … Parola, M. (2006). Dose dependent and divergent effects of superoxide anion on cell death, proliferation, and migration of activated human hepatic stellate cells. *Gut*, *55*(1), 90–97. https://doi.org/10.1136/gut.2005.069633

Novo, E., & Parola, M. (2008). Redox mechanisms in hepatic chronic wound healing and fibrogenesis. *Fibrogenesis & Tissue Repair*, *1*(1), 5. https://doi.org/10.1186/1755-1536-1-5

Okazaki, I., & Inagaki, Y. (2012). Novel strategies for hepatocellular carcinoma based on MMPs science. *Anti-Cancer Agents in Medicinal Chemistry*, *12*(7), 753–63. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/22292750

Ortmann, B., Druker, J., & Rocha, S. (2014). Cell cycle progression in response to oxygen levels. *Cellular and Molecular Life Sciences*, *71*(18), 3569–3582. https://doi.org/10.1007/s00018-014-1645-9

Parola, M., & Marra, F. (2011). Adipokines and Redox Signaling: Impact on Fatty Liver Disease. https://doi.org/10.1089/ars.2010.3848

Parola, M., Marra, F., & Pinzani, M. (2008). Myofibroblast – like cells and liver fibrogenesis: Emerging concepts in a rapidly moving scenario. *Molecular Aspects of Medicine*, *29*(1–2), 58–66. https://doi.org/10.1016/j.mam.2007.09.002

Parola, M., Robino, G., Marra, F., Pinzani, M., Bellomo, G., Leonarduzzi, G., … Dianzani, M. U. (1998). HNE interacts directly with JNK isoforms in human hepatic stellate cells. *The Journal of Clinical Investigation*, *102*(11), 1942–50. https://doi.org/10.1172/JCI1413

Pellicoro, A., Ramachandran, P., Iredale, J. P., & Fallowfield, J. A. (2014). Liver fibrosis and repair: immune regulation of wound healing in a solid organ. *Nature Reviews Immunology*, *14*(3), 181–194. https://doi.org/10.1038/nri3623

Pérez-Carreras, M., Del Hoyo, P., Martín, M. A., Rubio, J. C., Martín, A., Castellano, G., … Solis-Herruzo, J. A. (2003). Defective hepatic mitochondrial respiratory chain in patients with nonalcoholic steatohepatitis. *Hepatology*, *38*(4), 999–1007. https://doi.org/10.1053/jhep.2003.50398

Petta, S., Gastaldelli, A., Rebelos, E., Bugianesi, E., Messa, P., Miele, L., … Bonino, F. (2016). Pathophysiology of Non Alcoholic Fatty Liver Disease. *International Journal of Molecular Sciences*, *17*(12), 2082. https://doi.org/10.3390/ijms17122082

Peyssonnaux, C., Cejudo-Martin, P., Doedens, A., Zinkernagel, A. S., Johnson, R. S., & Nizet, V. (2007). Cutting edge: Essential role of hypoxia inducible factor-1alpha in development of lipopolysaccharide-induced sepsis. *Journal of Immunology (Baltimore, Md. : 1950)*, *178*(12), 7516–9. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/17548584

Peyssonnaux, C., Datta, V., Cramer, T., Doedens, A., Theodorakis, E. A., Gallo, R. L., … Johnson, R. S. (2005). HIF-1α expression regulates the bactericidal capacity of phagocytes. *Journal of Clinical Investigation*, *115*(7), 1806–1815. https://doi.org/10.1172/JCI23865

Pinzani, M., & Marra, F. (2001). Cytokine Receptors and Signaling in Hepatic Stellate Cells. *Seminars in Liver Disease*, *21*(3), 397–416. https://doi.org/10.1055/s-2001-17554

Pontisso. (2009). Role of squamous cell carcinoma antigen-1 on liver cells after partial hepatectomy in transgenic mice. *International Journal of Molecular Medicine*, *25*(1). https://doi.org/10.3892/ijmm_00000323

Pontisso, P. (2014). Role of SERPINB3 in hepatocellular carcinoma. *Annals of Hepatology*, *13*(6), 722–727. Retrieved from http://www.annalsofhepatology.com/revista/numeros/2014/03_146_v13n6_2014_RoleSERPINB3.pdf

Povero, D., Busletta, C., Novo, E., di Bonzo, L. V., Cannito, S., Paternostro, C., & Parola, M. (2010). Liver fibrosis: a dynamic and potentially reversible process. *Histology and Histopathology*, *25*(8), 1075–91. https://doi.org/10.14670/HH-25.1075

Prabhakar, N. R., & Semenza, G. L. (2015). Oxygen Sensing and Homeostasis. *Physiology (Bethesda, Md.)*, *30*(5), 340–8. https://doi.org/10.1152/physiol.00022.2015

Qu, A., Taylor, M., Xue, X., Matsubara, T., Metzger, D., Chambon, P., … Shah, Y. M. (2011). Hypoxia-inducible transcription factor 2α promotes steatohepatitis through augmenting lipid accumulation, inflammation, and fibrosis. *Hepatology*, *54*(2), 472–483. https://doi.org/10.1002/hep.24400

Quarta, S., Vidalino, L., Turato, C., Ruvoletto, M., Calabrese, F., Valente, M., … Pontisso, P. (2010). SERPINB3 induces epithelial-mesenchymal transition. *The Journal of Pathology*, *221*(3), 343–356. https://doi.org/10.1002/path.2708

Ramakrishnan, S. K., Zhang, H., Takahashi, S., Centofanti, B., Periyasamy, S., Weisz, K., … Shah, Y. M. (2016). HIF2α Is an Essential Molecular Brake for Postprandial Hepatic Glucagon Response Independent of Insulin Signaling. *Cell Metabolism*, *23*(3), 505–16. https://doi.org/10.1016/j.cmet.2016.01.004

Ramírez-Bergeron, D. L., Runge, A., Adelman, D. M., Gohil, M., & Simon, M. C. (2006). HIF-Dependent Hematopoietic Factors Regulate the Development of the Embryonic Vasculature. *Developmental Cell*, *11*(1), 81–92. https://doi.org/10.1016/j.devcel.2006.04.018

Rankin, E. B., Rha, J., Selak, M. A., Unger, T. L., Keith, B., Liu, Q., & Haase, V. H. (2009). Hypoxia-inducible factor 2 regulates hepatic lipid metabolism. *Molecular and Cellular Biology*, *29*(16), 4527–38. https://doi.org/10.1128/MCB.00200-09

Raval, R. R., Lau, K. W., Tran, M. G. B., Sowter, H. M., Mandriota, S. J., Li, J.-L., … Ratcliffe, P. J. (2005). Contrasting properties of hypoxia-inducible factor 1 (HIF-1) and HIF-2 in von Hippel-Lindau-associated renal cell carcinoma. *Molecular and Cellular Biology*, *25*(13), 5675–86. https://doi.org/10.1128/MCB.25.13.5675-5686.2005

Ripoli, M., D'Aprile, A., Quarato, G., Sarasin-Filipowicz, M., Gouttenoire, J., Scrima, R., … Piccoli, C. (2010). Hepatitis C Virus-Linked Mitochondrial Dysfunction Promotes Hypoxia-Inducible Factor 1 -Mediated Glycolytic Adaptation. *Journal of Virology*, *84*(1), 647–660. https://doi.org/10.1128/JVI.00769-09

Rius, J., Guma, M., Schachtrup, C., Akassoglou, K., Zinkernagel, A. S., Nizet, V., … Karin, M. (2008). NF-κB links innate immunity to the hypoxic response through transcriptional regulation of HIF-1α. *Nature*, *453*(7196), 807–811. https://doi.org/10.1038/nature06905

Ronca, F., & Raggi, A. (2015). Structure-function relationships in mammalian histidine-proline-rich glycoprotein. *Biochimie*, *118*, 207–220. https://doi.org/10.1016/j.biochi.2015.09.024

Roth, K. J., & Copple, B. L. (2015). Role of Hypoxia-Inducible Factors in the Development of Liver Fibrosis. *Cellular and Molecular Gastroenterology and Hepatology*, *1*(6), 589–597. https://doi.org/10.1016/j.jcmgh.2015.09.005

Roychowdhury, S., Chiang, D. J., McMullen, M. R., & Nagy, L. E. (2014). Moderate, chronic ethanol feeding exacerbates carbon-tetrachloride-induced hepatic fibrosis via hepatocyte-specific hypoxia inducible factor 1α. *Pharmacology Research & Perspectives*, *2*(5), e00061. https://doi.org/10.1002/prp2.61

Sahai, A., Malladi, P., Pan, X., Paul, R., Melin-Aldana, H., Green, R. M., & Whitington, P. F. (2004). Obese and diabetic db/db mice develop marked liver fibrosis in a model of nonalcoholic steatohepatitis: role of short-form leptin receptors and osteopontin. *AJP: Gastrointestinal and Liver Physiology*, *287*(5), G1035–G1043. https://doi.org/10.1152/ajpgi.00199.2004

Sakaida, I., Matsumura, Y., Akiyama, S., Hayashi, K., Ishige, A., & Okita, K. (1998). Herbal medicine Sho-saiko-to (TJ-9) prevents liver fibrosis and enzyme-altered lesions in rat liver cirrhosis induced by a choline-deficient □-amino acid-defined diet. *Journal of Hepatology*, *28*, 298–306. Retrieved from http://www.journal-of-hepatology.eu/article/0168-8278(88)80017-5/pdf

Samanta, D., Prabhakar, N. R., & Semenza, G. L. (2017). Systems biology of oxygen homeostasis. *Wiley Interdisciplinary Reviews: Systems Biology and Medicine*, *9*(4), e1382. https://doi.org/10.1002/wsbm.1382

Sanyal, A. J., & EPA-E investigators. (2015). Reply. *Gastroenterology*, *148*(1), 262–263. https://doi.org/10.1053/j.gastro.2014.11.030

Sasse, D., Spornitz, U. M., & Piotr Maly, I. (1992). Liver Architecture. *Enzyme*, *46*(1–3), 8–32. https://doi.org/10.1159/000468776

Schröder, M., & Sutcliffe, L. (2010). Consequences of Stress in the Secretary Pathway: The ER Stress Response and Its Role in the Metabolic Syndrome. In *Methods in molecular biology (Clifton, N.J.)* (Vol. 648, pp. 43–62). https://doi.org/10.1007/978-1-60761-756-3_3

Schütte, K., Schulz, C., Link, A., & Malfertheiner, P. (2015). Current biomarkers for hepatocellular carcinoma: Surveillance, diagnosis and prediction of prognosis. *World Journal of Hepatology*, *7*(2), 139–49. https://doi.org/10.4254/wjh.v7.i2.139

Scott, C., Cha, K., Rao, R., Liddle, C., George, J., & Gunton, J. E. (2015). Hepatocyte- Specific Deletion of ARNT (Aryl Hydrocarbon Receptor Nuclear Translocator) Results in Altered Fibrotic Gene Expression in the Thioacetamide Model of Liver Injury. *PLOS ONE*, *10*(3), e0121650. https://doi.org/10.1371/journal.pone.0121650

Seki, E., De Minicis, S., Gwak, G.-Y., Kluwe, J., Inokuchi, S., Bursill, C. A., … Schwabe, R. F. (2009). CCR1 and CCR5 promote hepatic fibrosis in mice. *The Journal of Clinical Investigation*, *119*(7), 1858–70. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19603542

Semenza, G. L. (2003). Targeting HIF-1 for cancer therapy. *Nature Reviews Cancer*, *3*(10), 721–732. https://doi.org/10.1038/nrc1187

Semenza, G. L. (2007). Life with oxygen. *Science (New York, N.Y.)*, *318*(5847), 62–64. https://doi.org/10.1126/science.1147949

Semenza, G. L. (2012). Hypoxia-inducible factors in physiology and medicine. *Cell*. https://doi.org/10.1016/j.cell.2012.01.021

Shay, J. E. S., & Celeste Simon, M. (2012, June). Hypoxia-inducible factors: Crosstalk between inflammation and metabolism. *Seminars in Cell and Developmental Biology*. https://doi.org/10.1016/j.semcdb.2012.04.004

Singh, R., Wang, Y., Xiang, Y., Tanaka, K. E., Gaarde, W. A., & Czaja, M. J. (2009). Differential effects of JNK1 and JNK2 inhibition on murine steatohepatitis and insulin resistance. *Hepatology (Baltimore, Md.)*, *49*(1), 87–96. https://doi.org/10.1002/hep.22578

Skuli, N., & Simon, M. C. (2009). HIF-1alpha versus HIF-2alpha in endothelial cells and vascular functions: is there a master in angiogenesis regulation? *Cell Cycle (Georgetown, Tex.)*, *8*(20), 3252–3. https://doi.org/10.4161/cc.8.20.9618

Sterling, R. K., Wright, E. C., Morgan, T. R., Seeff, L. B., Hoefs, J. C., Di Bisceglie, A. M., … Lok, A. S. (2012). Frequency of elevated hepatocellular carcinoma (HCC) biomarkers in patients with advanced hepatitis C. *The American Journal of Gastroenterology*, *107*(1), 64–74. https://doi.org/10.1038/ajg.2011.312

Sun, H.-X., Xu, Y., Yang, X.-R., Wang, W.-M., Bai, H., Shi, R.-Y., … Fan, J. (2013). Hypoxia inducible factor 2 alpha inhibits hepatocellular carcinoma growth through the transcription factor dimerization partner 3/ E2F transcription factor 1-dependent apoptotic pathway. *Hepatology (Baltimore, Md.)*, *57*(3), 1088–97. https://doi.org/10.1002/hep.26188

Sutti, S., Bruzzì, S., & Albano, E. (2016). The role of immune mechanisms in alcoholic and nonalcoholic steatohepatitis: a 2015 update. *Expert Review of Gastroenterology & Hepatology*, *10*(2), 243–253. https://doi.org/10.1586/17474124.2016.1111758

Sutti, S., Locatelli, I., Bruzzì, S., Jindal, A., Vacchiano, M., Bozzola, C., & Albano, E. (2015). CX3CR1-expressing inflammatory dendritic cells contribute to the progression of steatohepatitis. *Clinical Science (London, England : 1979)*, *129*(9), 797–808. https://doi.org/10.1042/CS20150053

Szczepaniak, L. S., Nurenberg, P., Leonard, D., Browning, J. D., Reingold, J. S., Grundy, S., … Dobbins, R. L. (2005). Magnetic resonance spectroscopy to measure hepatic triglyceride content: prevalence of hepatic steatosis in the general population. *American Journal of Physiology. Endocrinology and Metabolism*, *288*(2), E462-8. https://doi.org/10.1152/ajpendo.00064.2004

Takeda, N., O'Dea, E. L., Doedens, A., Kim, J. -w., Weidemann, A., Stockmann, C., … Johnson, R. S. (2010). Differential activation and antagonistic function of HIF- isoforms in macrophages are essential for NO homeostasis. *Genes & Development*, *24*(5), 491–501. https://doi.org/10.1101/gad.1881410

Talks, K. L., Turley, H., Gatter, K. C., Maxwell, P. H., Pugh, C. W., Ratcliffe, P. J., & Harris, A. L. (2000). The expression and distribution of the hypoxia-inducible factors HIF-1alpha and HIF-2alpha in normal human tissues, cancers, and tumor-associated macrophages. *The American Journal of Pathology*, *157*(2), 411–21. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/10934146

Taniguchi, C. M., Finger, E. C., Krieg, A. J., Wu, C., Diep, A. N., LaGory, E. L., … Giaccia, A. J. (2013). Cross-talk between hypoxia and insulin signaling through Phd3 regulates hepatic glucose and lipid metabolism and ameliorates diabetes. *Nature Medicine*, *19*(10), 1325–30. https://doi.org/10.1038/nm.3294

Tannahill, G. M., Curtis, A. M., Adamik, J., Palsson-McDermott, E. M., McGettrick, A. F., Goel, G., … O'Neill, L. A. J. (2013). Succinate is an inflammatory signal that induces IL-1β through HIF-1α. *Nature*, *496*(7444), 238–42. https://doi.org/10.1038/nature11986

Taylor, C. T., Doherty, G., Fallon, P. G., & Cummins, E. P. (2016). Hypoxia-dependent regulation of inflammatory pathways in immune cells. *The Journal of Clinical Investigation*, *126*(10), 3716–3724. https://doi.org/10.1172/JCI84433

Thompson, A. A. R., Elks, P. M., Marriott, H. M., Eamsamarng, S., Higgins, K. R., Lewis, A., … Walmsley, S. R. (2014). Hypoxia-inducible factor 2 regulates key neutrophil functions in humans, mice, and zebrafish. *Blood*, *123*(3), 366–376. https://doi.org/10.1182/blood-2013-05-500207

Tilg, H., & Moschen, A. R. (2010). Evolution of inflammation in nonalcoholic fatty liver disease: The multiple parallel hits hypothesis. *Hepatology*, *52*(5), 1836–1846. https://doi.org/10.1002/hep.24001

Tillmanns, J., Rota, M., Hosoda, T., Misao, Y., Esposito, G., Gonzalez, A., … Anversa, P. (2008).

Formation of large coronary arteries by cardiac progenitor cells. *Proceedings of the National Academy of Sciences of the United States of America*, *105*(5), 1668–73. https://doi.org/10.1073/pnas.0706315105

Tsukada, K., Tajima, T., Hori, S., Matsuura, T., Johnson, R. S., Goda, N., & Suematsu, M. (2013). Hypoxia-Inducible Factor-1 Is a Determinant of Lobular Structure and Oxygen Consumption in the Liver. *Microcirculation*, *20*(5), 385–393. https://doi.org/10.1111/micc.12033

Turato, C., Calabrese, F., Biasiolo, A., Quarta, S., Ruvoletto, M., Tono, N., … Pontisso, P. (2010). SERPINB3 modulates TGF-β expression in chronic liver disease. *Laboratory Investigation*, *9055*, 1016–1023. https://doi.org/10.1038/labinvest.2010.55

Turato, C., Vitale, A., Fasolato, S., Ruvoletto, M., Terrin, L., Quarta, S., … Pontisso, P. (2014). SERPINB3 is associated with TGF-β1 and cytoplasmic β-catenin expression in hepatocellular carcinomas with poor prognosis. *British Journal of Cancer*, *110*(11), 2708–15. https://doi.org/10.1038/bjc.2014.246

Unger, R. H. (2002). Lipotoxic diseases. *Annual Review of Medicine*, *53*(1), 319–36. https://doi.org/10.1146/annurev.med.53.082901.104057

Valfrè di Bonzo, L., Novo, E., Cannito, S., Busletta, C., Paternostro, C., Povero, D., & Parola, M. (2009). Angiogenesis and liver fibrogenesis. *Histology and Histopathology*, *24*(10), 1323–41. https://doi.org/10.14670/HH-24.1323

Vance, J. E., & Vance, D. E. (1985). The role of phosphatidylcholine biosynthesis in the secretion of lipoproteins from hepatocytes. *Canadian Journal of Biochemistry and Cell Biology = Revue Canadienne de Biochimie et Biologie Cellulaire*, *63*(8), 870–81. https://doi.org/10.1139/o85-108

Villano, G., Quarta, S., Ruvoletto, M. G., Turato, C., Vidalino, L., Biasiolo, A., … Pontisso, P. (2010). Role of squamous cell carcinoma antigen-1 on liver cells after partial hepatectomy in transgenic mice. *International Journal of Molecular Medicine*, *25*(1), 137–43. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/19956912

Vollmers, H. P., & Brändlein, S. (2009). Natural antibodies and cancer. *New Biotechnology*, *25*(5), 294–298. https://doi.org/10.1016/j.nbt.2009.03.016

Wan, H.-G., Xu, H., Gu, Y.-M., Wang, H., Xu, W., & Zu, M.-H. (2014). Comparison osteopontin vs AFP for the diagnosis of HCC: a meta-analysis. *Clinics and Research in Hepatology and Gastroenterology*, *38*(6), 706–14. https://doi.org/10.1016/j.clinre.2014.06.008

Wang, L., Potter, J. J., Rennie-Tankersley, L., Novitskiy, G., Sipes, J., & Mezey, E. (2007). Effects of retinoic acid on the development of liver fibrosis produced by carbon tetrachloride in mice. *Biochimica et Biophysica Acta (BBA) - Molecular Basis of Disease*, *1772*(1), 66–71. https://doi.org/10.1016/j.bbadis.2006.08.009

Wang, X. L., Suzuki, R., Lee, K., Tran, T., Gunton, J. E., Saha, A. K., … Kahn, C. R. (2009). Ablation of ARNT/HIF1beta in liver alters gluconeogenesis, lipogenic gene expression, and serum ketones. *Cell Metabolism*, *9*(5), 428–39. https://doi.org/10.1016/j.cmet.2009.04.001

Webby, C. J., Wolf, A., Gromak, N., Dreger, M., Kramer, H., Kessler, B., … Böttger, A. (2009). Jmjd6 Catalyses Lysyl-Hydroxylation of U2AF65, a Protein Associated with RNA Splicing. *Science*, *325*(5936), 90–93. https://doi.org/10.1126/science.1175865

Wei, K., Piecewicz, S. M., McGinnis, L. M., Taniguchi, C. M., Wiegand, S. J., Anderson, K., … Kuo, C. J. (2013). A liver Hif-2α-Irs2 pathway sensitizes hepatic insulin signaling and is modulated by Vegf inhibition. *Nature Medicine*, *19*(10), 1331–1337. https://doi.org/10.1038/nm.3295

Wells, R. G. (2014). The portal fibroblast: not just a poor man's stellate cell. *Gastroenterology*,

147(1), 41–7. https://doi.org/10.1053/j.gastro.2014.05.001

Weng, H.-L., Liu, Y., Chen, J.-L., Huang, T., Xu, L.-J., Godoy, P., … Dooley, S. (2009). The etiology of liver damage imparts cytokines transforming growth factor beta1 or interleukin-13 as driving forces in fibrogenesis. *Hepatology (Baltimore, Md.)*, *50*(1), 230–43. https://doi.org/10.1002/hep.22934

Wilson, G. K., Brimacombe, C. L., Rowe, I. A., Reynolds, G. M., Fletcher, N. F., Stamataki, Z., … McKeating, J. A. (2012). A dual role for hypoxia inducible factor-1α in the hepatitis C virus lifecycle and hepatoma migration. *Journal of Hepatology*, *56*(4), 803–9. https://doi.org/10.1016/j.jhep.2011.11.018

Wilson, G. K., Tennant, D. A., & McKeating, J. A. (2014). Hypoxia inducible factors in liver disease and hepatocellular carcinoma: current understanding and future directions. *Journal of Hepatology*, *61*(6), 1397–406. https://doi.org/10.1016/j.jhep.2014.08.025

Xia, L., Mo, P., Huang, W., Zhang, L., Wang, Y., Zhu, H., … Wu, K. (2012). The TNF-α/ROS/HIF-1-induced upregulation of FoxMI expression promotes HCC proliferation and resistance to apoptosis. *Carcinogenesis*, *33*(11), 2250–9. https://doi.org/10.1093/carcin/bgs249

Xiang, Z.-L., Zeng, Z.-C., Fan, J., Tang, Z.-Y., He, J., Zeng, H.-Y., & Chang, J. Y. (2012). The expression of HIF-1α in primary hepatocellular carcinoma and its correlation with radiotherapy response and clinical outcome. *Molecular Biology Reports*, *39*(2), 2021–2029. https://doi.org/10.1007/s11033-011-0949-1

Yamane, K., Toumazou, C., Tsukada, Y., Erdjument-Bromage, H., Tempst, P., Wong, J., & Zhang, Y. (2006). JHDM2A, a JmjC-containing H3K9 demethylase, facilitates transcription activation by androgen receptor. *Cell*, *125*(3), 483–95. https://doi.org/10.1016/j.cell.2006.03.027

Yang, L., Kwon, J., Popov, Y., Gajdos, G. B., Ordog, T., Brekken, R. A., … Shah, V. H. (2014). Vascular endothelial growth factor promotes fibrosis resolution and repair in mice. *Gastroenterology*, *146*(5), 1339–50.e1. https://doi.org/10.1053/j.gastro.2014.01.061

Yoo, Y.-G., Oh, S. H., Park, E. S., Cho, H., Lee, N., Park, H., … Lee, M.-O. (2003). Hepatitis B virus X protein enhances transcriptional activity of hypoxia-inducible factor-1alpha through activation of mitogen-activated protein kinase pathway. *The Journal of Biological Chemistry*, *278*(40), 39076–84. https://doi.org/10.1074/jbc.M305101200

Younossi, Z., Anstee, Q. M., Marietti, M., Hardy, T., Henry, L., Eslam, M., … Bugianesi, E. (2017). Global burden of NAFLD and NASH: trends, predictions, risk factors and prevention. *Nature Reviews Gastroenterology & Hepatology*, nrgastro.2017.109. https://doi.org/10.1038/nrgastro.2017.109

Zamara, E., Novo, E., Marra, F., Gentilini, A., Romanelli, R. G., Caligiuri, A., … Parola, M. (2004). 4-Hydroxynonenal as a selective pro-fibrogenic stimulus for activated human hepatic stellate cells. *Journal of Hepatology*, *40*(1), 60–8. Retrieved from http://www.ncbi.nlm.nih.gov/pubmed/14672615

Zhang, Q., Jiang, K., Li, Y., Gao, D., Sun, L., Zhang, S., … Liu, Y. (2015). Histidine-rich glycoprotein function in hepatocellular carcinoma depends on its N-glycosylation status, and it regulates cell proliferation by inhibiting Erk1/2 phosphorylation. *Oncotarget*, *6*(30), 30222–30231. https://doi.org/10.18632/oncotarget.4997

Zhao, S., Xu, W., Jiang, W., Yu, W., Lin, Y., Zhang, T., … Guan, K.-L. (2010). Regulation of Cellular Metabolism by Protein Lysine Acetylation. *Science*, *327*(5968), 1000–1004. https://doi.org/10.1126/science.1179689

Zhu, P., Huang, L., Ge, X., Yan, F., Wu, R., & Ao, Q. (2006). Transdifferentiation of pulmonary

arteriolar endothelial cells into smooth muscle-like cells regulated by myocardin involved in hypoxia-induced pulmonary vascular remodelling. *International Journal of Experimental Pathology*, *87*(6), 463–474. https://doi.org/10.1111/j.1365-2613.2006.00503.x