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MicroRNAs in liver disease

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ABBREVIATIONS

ACC: acetyl coenzyme A carboxylase.

Acetyl CoA: acetyl coenzyme A.

ALT: alanine aminotransferase.

AMPK: AMP activated protein kinase.

ApoB: apolipoprotein B 100.

AST: aspartate transaminase.

ATP: adenosine triphosphate.

BA: bile acid.

BCLC: Barcelona clinic liver cancer.

BDL: bile duct ligation.

BHMT: betaine homocysteine S-methyltransferase.

BMI: body mass index.

BN-PAGE: blue native-polyacrylamide gel electrophoresis.

BSEP: bile salt export pump.

BrdU: bromodeoxyuridine.

CA: cholic acid.

CBS: cystathionine-beta-synthase.

CCL: C-C motif chemokine ligand.

CCR: C-C motif chemokine receptor.

CDCA: chenodeoxycholic acid.

ChREBP: Carbohydrate-responsive element-binding protein.

CPT: carnitine palmitoyltransferase.

CXCL: C-X-C motif chemokine ligand.

CXCR: C-X-C motif chemokine receptor.

CYPs: cytochrome P450.

Cyt *c*: cytochrome *c*.

DCA: deoxycholic acid.

DCR: disease control rate.

DGAT: diacylglycerol acyltransferase.

DMEM: Dulbecco’s modified eagle medium.

DNMT: DNA methyltransferase.

DR5: death receptor 5.

ECM: extracellular matrix.

EGF: epidermal growth factor

EGFR: epidermal growth factor receptor

EMT: epidermal mesenchymal transition.

ETC: electron transport chain.

ER: endoplasmic reticulum.

EZH2: enhancer of zeste homolog 2.

FA: fatty acid.

FAO: fatty acid oxidation.

FASN: fatty acid synthase.

FBS: fetal bovine serum.

FGF: fibroblast growth factor.

FGFR: fibroblast growth factor receptor.

FFA: free fatty acid.

FXR: farnesoid X receptor.

FD: fructose diet.

$FADH_2$/FAD: Flavin adenine dinucleotide.

GNMT: glycine N-methyltransferase.

Gnmt$^{-/-}$: *Gnmt*-knock out.

GSH: glutathione.

HAT: histone acetyltransferase.

HBV: hepatitis B virus.

HCC: hepatocellular carcinoma.

HCD: high cholesterol diet.

HCV: hepatitis C virus.

HCY: homocysteine.

HDAC: histone deacetylase.

HDL: high density lipoprotein.

HFD: high fat diet.

HSC: hepatic stellate cell.

IL-6: interleukin 6.

IP: immunoprecipitated protein.

i.v.: intravenously.

JAK: janus kinase.

JNK: c-Jun N-terminal kinase.

KB: ketone body.

KC: Kupffer cell.

LCA: lithocholic acid.

LDL: low density lipoprotein.

LC-MS: liquid chromatography-mass spectrometry.

MAT: methionine adenosyltransferase.

MeCP2: Methyl-CpG Binding Protein 2.

MBD: methyl binding domain.

MCDD: methionine choline deficient diet.

MCP-1: monocyte chemoattractant protein-1.

MDMC: medium deficient in methionine and choline.

MDR: multi drug resistance.

MEM: minimum essential medium.

MiR, miRNA: microRNA.

MMP: metalloproteinase.

MOC: mechanisms of chemoresistance.

MRP: multidrug resistance associated protein.

MS: methionine synthase.

MTA: 5'-methylthioadenisine.

MTHFR: methylenetetrahydrofolate reductase.

MTHFS: methenyltetrahydrofolate synthetase.

mTOR: mammalian target of rapamycin.

MVUH: Marqués de Valdecilla University Hospital

NADH/NAD^+: nicotinamide adenine dinucleotide.

NAFL: non-alcoholic fatty liver.

NAFLD: non-alcoholic fatty liver disease.

NASH: non-alcoholic steatohepatitis.

NF-κB: nuclear factor kappa-light-chain-enhancer of activated B cells.

NK: natural killer.

NOS: nitric oxide synthase.

NPC2: Niemann-Pick type C2 protein.

NT: non-tumor.

OA: oleic acid.

OCR: oxygen consumption rate.

OS: overall survival.

OXPHOS: oxidative phosphorylation.

PBC: primary biliary cholangitis; primary biliary cirrhosis.

PBS: Phosphate buffered saline.

PC: phosphatidylcholine.

PCR: polymerase chain reaction.

PDGF: platelet-derived growth factor

PDGFR: platelet-derived growth factor receptor.

PE: phosphatidylethanolamine.

PEMT: phosphatidylethanolamine N-methyltransferase.

PIAS: protein inhibitor of activated STAT.

PPAR: peroxisome proliferator-activated receptors.

PSC: primary sclerosing cholangitis.

PSG: penicillin, streptomycin and glutamine.

PUMA: P53 upregulated modulator of apoptosis.

RNA: ribonucleic acid.

ROS: reactive oxygen species.

RT: room temperature.

RT qPCR: quantitative real-time reverse transcription polymerase chain reaction.

RTS: radiological time of progression.

SAH: S-adenosylhomocysteine.

SAHH: S-adenosylhomocysteine hydrolase.

SAMe: S-adenosylmethionine.

SDH: succinate dehydrogenase.

Sf: sorafenib.

SHARP: sorafenib hepatocellular carcinoma assessment randomized protocol.

SHP: small heterodimer partner.

SiRNA: small interfering RNA.

SLC: solute carrier.

SOCS: suppressor of cytokine signaling.

SOD: superoxide dismutase.

SREBP-1c: sterol regulatory element-binding protein 1.

STAT: signal transducers and activators of transcription.

T: tumor.

TACE: transarterial chemoembolization.

TCA: tricarboxylic acid.

TET: ten-eleven translocation.

TG: triglyceride.

TGFβ: transforming growth factor beta.

TIMP: tissue inhibitor of metalloproteinase.

TKR: tyrosine kinase receptor.

TNF: tumor necrosis factor.

TRAIL: TNF-related apoptosis inducing ligand.

TTSP: time to symptomatic progression.

UN: Universidad de Navarra.

VEGF: vascular endothelial growth factor.

VEGFR: vascular endothelial growth factor receptor.

VLCFA: very long-chain fatty acid.

VLDL: very low-density lipoprotein.

αSMA: alpha smooth muscle actin.

5mC: 5-methylcytosine.

5hmC: 5-hydroxymethylcitosine.

1 SUMMARY

SUMMARY

Chronic liver disease refers to a large group of pathologies generally characterized by a slow progression and the final development of cirrhosis and hepatocellular carcinoma. It can be caused by several damaging agents that affect liver function, such as alcohol and drug abuse, hepatitis virus infection, autoimmune and hereditary disorders and non-alcoholic fatty liver disease. Chronic liver disease is one of the most frequent cause of mortality in the United States and Europe. The increasing amount of emerging risk factors associated with chronic liver disease and the lack of effective therapies to treat it highlight the necessity of the better characterization of the molecular mechanism underlying this disease.

During the last years, increasing evidence have indicated dysregulations of methionine and S-adenosylmethionine (SAMe) metabolism and of those enzymes participating in the methionine cycle are implicated in different manifestations of chronic liver disease. Studies carried out by our group and others have identified frequent downregulation of Glycine N-methyltransferase (GNMT), the most important enzyme implicated in SAMe catabolism, in liver pathologies such as NAFLD, cholestasis, fibrosis, cirrhosis and hepatocellular carcinoma. These studies have revealed GNMT deficiency as an important mechanism driving liver disease and affecting several hepatic functions. Despite the importance of GNMT for normal liver function avoiding liver disease development, the mechanisms mediating GNMT downregulation have been poorly addressed and present several limitations concerning their implication in liver disease.

Parallel, the recent discovery of the microRNAs has led to understand many biological processes and diseases. MicroRNAs are small non-coding RNAs that regulate gene expression at the posttranscriptional level by mRNA targeting and repression. Since the microRNAs discovery, the expression of many miRNAs has been described deregulated in liver disease, contributing to understand liver pathobiology and emerging as new targets for the design of liver therapies.

The main objective of this project is to study the general mechanism mediating GNMT downregulation in different chronic liver disease scenarios: NAFLD, cholestasis and fibrosis and HCC. We hypothesize that GNMT is targeted and repressed by dysregulated microRNAs in the liver.

NAFLD is one of the most frequent chronic liver diseases in develop countries, where it is associated with metabolic syndrome risk factors and can range from simple steatosis (lipid accumulation) to steatohepatitis (NASH). GNMT is frequently downregulated in NAFLD patients and murine models and the *Gnmt*$^{-/-}$ mouse spontaneously develops hepatic steatosis and steatohepatitis without body mass gain. We show miR-873-5p is upregulated in human NAFLD patients and in murine dietary models, correlating GNMT downregulation. Inhibition of miR-873-5p results in normal expression of GNMT in the liver and in concrete in the mitochondrion. Mitochondrial GNMT levels are essential for the maintenance of SDH Complex II activity in the ETC, avoiding ROS generation, mitochondrial dysfunction and reduction in fatty acid β-oxidation and OXPHOS.

We have further investigated the role of miR-873-5p in cholestasis, fibrosis and cirrhosis, diseases characterized by GNMT downregulation and aggravated in the absence of this enzyme. MiR-873-5p upregulation correlates with GNMT downregulation in human and murine fibrotic models. Anti-miR-873-5p treatment in mice inhibits miR-873-5p mediated repression of GNMT. Recovery of GNMT in hepatocytes and other hepatic cell types protects from hepatocyte apoptosis, ductular proliferation, inflammation and fibrogenesis. Normal GNMT levels restores SAMe metabolism, avoiding aberrant DNA and histone methylation, highlighting the importance of maintaining homeostatic methionine and SAMe metabolism in the liver.

Chronic liver disease can evolve to hepatocellular carcinoma (HCC). HCC is the fifth most common cancer and the second cause of cancer related death, due to the high heterogeneity of the tumor and the presence of several molecular pathways converging within the tumor. HCC is a very poor prognosis cancer with difficult treatment and low success of the unique anticancer drug approved for systemic therapy (sorafenib), due to the above mentioned characteristics that frequently leads to drug resistance. Upregulation of miR-518d-5p correlates with GNMT downregulation in HCC. We have demonstrated that serum miR-518d-5p levels are increased in patients considered as non responders to sorafenib treatment in a prospective cohort. Therefore miR-518d-5p appears as a predictor biomarker for Sorafenib response. Sorafenib resistance in hepatoma cells is resolved by miR-518d-5p inhibition, which results in GNMT and c-Jun upregulation, both of them direct targets of the microRNA. MiR-518d-5p regulation of sorafenib induced apoptosis is mediated by increasing ROS and mitochondrial dysfunction.

In conclusion our results show the importance of the GNMT in the maintenance of liver health. The microRNAs miR-873-5p and miR-518d-5p are implicated in GNMT downregulation in different stages of chronic liver disease, affecting different cellular processes and emerging as interesting therapeutical targets.

2 INTRODUCTION

2 INTRODUCTION

2.1 CHRONIC LIVER DISEASE

Chronic liver disease is a term that includes a broad group of hepatic pathologies from different etiology that last longer than 6 months and are commonly ended in cirrhosis and hepatocellular carcinoma (HCC) (Mishra and Younossi, 2012; Riley and Bhatti, 2001; Vernon et al., 2011). Chronic liver disease is one of the leading cause of mortality in the United States and Europe and it can be caused by different pathologies, including viral infection of hepatitis B and C, toxins, alcohol and drug abuse, some autoimmune liver disease (primary sclerosis cholangitis and primary biliary cirrhosis), hereditary diseases and Non-Alcoholic Fatty Liver Disease (NAFLD) (Mishra and Younossi, 2012; Riley and Bhatti, 2001; Vernon et al., 2011). Despite the different etiology of these pathologies termed as chronic liver disease, most of them are characterized by a slow progression, frequently over 20 to 40 years, from hepatitis to cirrhosis and finally HCC (Riley and Bhatti, 2001).

2.1.1 NON-ALCOHOLIC FATTY LIVER DISEASE

NAFLD is emerging as one of the most frequent causes of chronic liver disease worldwide (Vernon et al., 2011) and, particularly, as the main manifestation in Western countries, with and incidence of 20-30% in general population, becoming a major health problem in the world (Bellentani et al., 2010; Loomba and Sanyal, 2013). NAFLD is a clinical syndrome that includes a spectrum of hepatic disorders ranging from simple lipid accumulation within the hepatocytes (steatosis or non-alcoholic fatty liver; NAFL) to hepatic steatosis with inflammation and, occasionally, fibrosis (steatohepatitis or non-alcoholic steatohepatitis; NASH).

NAFLD is closely associated to obesity, type 2 diabetes, insulin resistance, dyslipidemia and hypertension (Adams and Lindor, 2007; Adams et al., 2005; Calzadilla Bertot and Adams, 2016; Loomba and Sanyal, 2013; Noureddin and Rinella, 2015; Teli et al., 1995; Utzschneider and Kahn, 2006; Vernon et al., 2011), all of them considered risk factors for the development of metabolic syndrome (Siegel and Zhu, 2009). Moreover, NAFLD prevalence (accounted for a 20-30% in general population in western countries) is increased to 30-50% in diabetic patients and presented in 80-90% in obese people, tuning almost universal when combining both factors (Bellentani et al., 2010). In the case of children, NAFLD prevalence has risen up from 3-10% to 40-70% (Bellentani

et al., 2010). The increase prevalence of NAFLD during the last years is expected to rise up in the near future as patients with metabolic syndrome are increased; hence, NAFLD represent an incoming global health problem (Loomba and Sanyal, 2013; Mishra and Younossi, 2012).

The progression of NAFLD requires a set of steps usually studied as the "two-hit hypothesis" (Day and James, 1998; Sanyal, 2005) (Figure 2.1). First, an initial "hit" in the liver is given to start a process of adipose tissue lipopisis leading to fatty acids (FAs) accumulation in the liver, developing steatosis/NAFL. Steatosis is frequently considered a benign disease with good prognosis that is commonly reversible by changing underlying causes of the disease like unhealthy lifestyle (Mishra and Younossi, 2012). Despite the good prognosis of steatosis, about a 10%-30% of the patients with simple steatosis progress NASH, with 20% of them developing cirrhosis within the next 10 years (Farrell and Larter, 2006; Harrison et al., 2003; Marrero et al., 2002) and, finally, liver failure and HCC (4-27%) (Takuma and Nouso, 2010). For this initial progression from steatosis to NASH, a second "hit" is required. Different possible second "hits" have been proposed by researchers, being the most commonly accepted the activation of endoplasmic reticulum (ER) stress and the increase oxidative stress by overproduction of reactive oxygen species (ROS) and/or decreased antioxidant defences (Day and James, 1998; Sanyal, 2005). The continue overproduction of ROS leads to mitochondrial dysfunction (Berson et al., 1998), release of proinflammatory cytokines (Day, 2006; Kershaw and Flier, 2004) and hepatocyte apoptosis , contributing to the development of hepatitis and fibrosis (Berson et al., 1998; Sanyal, 2005).

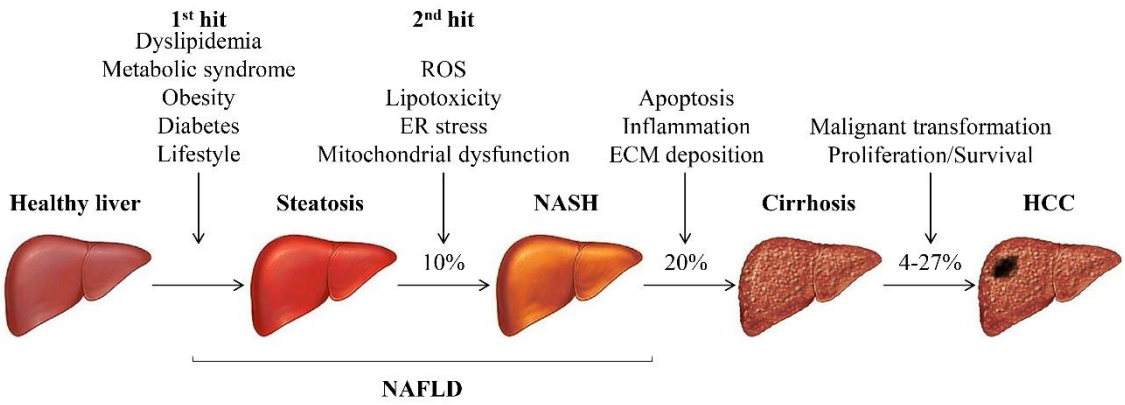

Figure 2.1. Liver disease progression from NAFLD to HCC. Steatosis develops as a consequence of lipid storage in the liver due to different causes (1st "hit"). 2nd "hit" in form of reactive oxygen species (ROS), lipotoxicity and endoplasmic reticulum stress can be presented in the liver, leading to non-alcoholic steatohepatitis (NASH) in 10%-30% of NAFLD patients. Sustained damage results in fibrotic

response and cirrhosis in 25% of patients. Finally, 4-27% of cirrhotic patients can develop hepatocellular carcinoma (HCC), the most common manifestation of liver cancer.

Despite the common use of this "two-hit" hypothesis to refeer to the progression of NAFLD, nowadays it is becoming more evident that different factors may converge at the same time synergistically contributing to the development of the disease, which is known as the "multiple-hit" hypothesis. In the next two sections the mechanisms implicated in the initiation (alterations in lipid homeostasis, "first hits" or "factors") and the progression (ROS production and mitochondrial dysfunction "second hits") required for the development of NAFLD will be described.

2.1.1.1 Alterations in lipid homeostasis

As mentioned before, hepatic steatosis is characterized by lipid accumulation in the liver that results from an imbalance between processes involved in production and turnover. Increased fatty acid uptake and/or de novo lipogenesis and defective triglyceride export and lipid degradation will lead to this lipid accumulation in the hepatocytes (Figure 2.2).

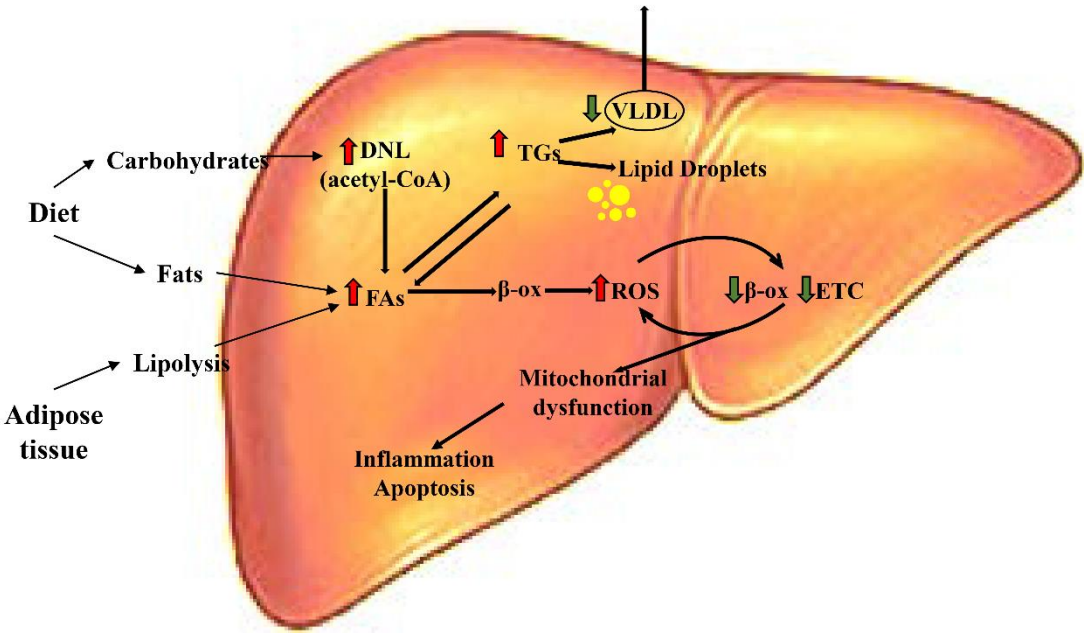

Figure 2.2. Main pathways implicated in triglyceride accumulation in the liver and disease progression. NAFLD is characterized by TG accumulation in lipid droplets. This can be the consequence of increased FA uptake from the diet or adipose tissue, enhanced lipogenesis in the liver, decreased VLDL secretion and impairment in β-oxidation. Lipid accumulation can predispose the liver to mitochondrial dysfunction, with increased ROS, which later promotes inflammation and apoptosis in hepatocytes, leading to disease progression (NASH).

2.1.1.1.1 Increased *de novo* lipogenesis and fatty acid uptake

De novo lipogenesis is a process regulated mainly by the enzymes acetyl coenzyme A (acetyl CoA) carboxylase (ACC) and fatty acid synthetase (FASN) that constitutes an important source of FAs in the liver. *De novo* lipogenesis contribution to hepatic TG content in normal individuals is estimated at less than 5% while it has been described to be increased in NAFLD patients accounting for 15-23%, even during fasting stages (Diraison et al., 2003; Donnelly et al., 2005; Lambert et al., 2014). In this context, nutrients coming from the diet are very relevant contributors to *de novo* lipogenesis in the liver. Not only FAs but also carbohydrates and fructose constitute important sources of FAs to the global liver pool.

Finally, the FA pool in the liver can be also altered by an excess of free fatty acids (FFAs) supply from the white adipose tissue. White adipose tissue is the major source of FA in the body. Under specific circumstance, TG contained in the adipose tissue are hydrolysed releasing FFAs that are delivered to the liver. This process has been found to be upregulated in NAFLD (Fabbrini et al., 2010).

De novo lipogenesis must be tightly regulated by several molecular mechanisms that implicates different enzymes (ACC1/2, FAS, stearoyl-CoA desaturase 1 (SCD1) and diacylglycerol acyltransferase (DGAT) 1/2)) involved in the conversion of acetyl CoA to palmitate and TG. These enzymes are, in turn, transcriptionally regulated by several transcription factors, particularly the sterol regulatory element-binding protein-1 isoform c (SREBP-1c) (Shimano et al., 1997; Shimomura et al., 1999) and the carbohydrate response element-binding protein (ChREBP) (Yamashita et al., 2001), stimulated by insulin and glucose, respectively. Other transcription factors implicated in *de novo* lipogenesis regulation are liver X receptor α (LXRα), farnesoid X receptor (FXR) and peroxisome proliferator-activated receptor γ (PPARγ)(Fabbrini et al., 2010; Sanyal, 2005; Strable and Ntambi, 2010) (Figure 2.3).

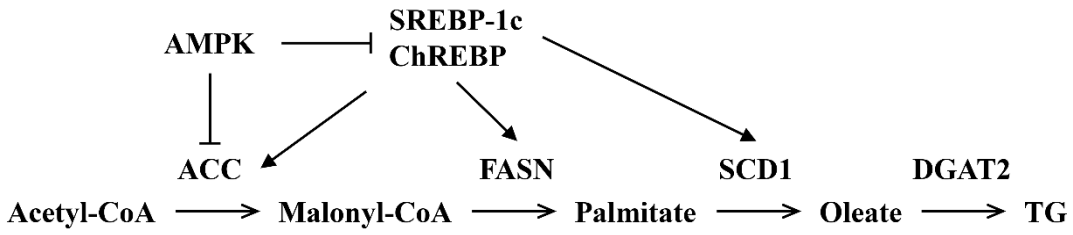

Figure 2.3. *De novo* lipogenesis. Principal steps implicated in TG synthesis from simple precursors (acetyl-CoA). *De novo* lipogenesis is frequently augmented in NAFLD, and it is controlled by different

transcription factors that regulates the expression of different enzymes implicated in different steps of lipogenesis.

2.1.1.1.2 Impaired VLDL secretion

Exceeding FAs in the liver are transformed to TGs, which can be stored or secreted into VLDL to the circulation for their delivery to peripheral tissues. VLDL are macromolecular complexes mainly formed by TGs and cholesteryl esters surrounded by an envelope of phospholipids and unesterified cholesterol, all stabilized by a molecule of apolipoprotein B 100 (apoB). Increased VLDL production is a common feature in NAFLD, however, the increased production of VLDL cannot compensate the increased TGs synthesis that is produced in the liver (Fabbrini et al., 2010; Kawano and Cohen, 2013). Moreover, it has been reported that oxidative stress and endoplasmic reticulum stress, both characteristics of NASH, contribute to the degradation of apoB by proteasomal and non-proteasomal mechanisms, impairing TG secretion from the liver and contributing to fatty liver (Ota et al., 2008; Pan et al., 2004).

2.1.1.1.3 Impaired fatty acid β-oxidation.

The liver has the ability to degrade FFAs through mitochondrial fatty acid β-oxidation (FAO) in a series of steps critical to produce energy in form of ATP and ketone bodies. FAO is regulated by different mechanism (Figure 2.4). First, carnitine palmitoyltransferases 1/2 (**CPT1**/2) are the limiting enzymes to translocate FAs into the mitochondrion. CPT1 is negatively regulated by malonyl CoA produced from acetyl CoA during *de novo* lipogenesis (Fabbrini et al., 2010). Thus, *de novo* lipogenesis can regulate FAO inhibiting the entrance of FAs to the mitochondrion. On the other hand, CPT1 can be positively regulated by PPARα, which promotes the transcription of malonyl CoA decarboxylase, implicated in the degradation of malonyl CoA (Lee et al., 2004). PPARα is also implicated in the regulation and transcription of most of the enzymes implicated in FAO, being a master regulator of mitochondrial β-oxidation (Mandard et al., 2004; Mello et al., 2016; Rakhshandehroo et al., 2010). Finally, FAO is regulated by AMPK phosphorylation, which inactivates *de novo* lipogenesis through ACC and SREBP1c phosphorylation and increases β-oxidation directly binding and activating PPARα. FAO is linked to other mitochondrial functions such as the TCA cycle and the electron transport chain (ETC), regulating the reduction power production in the mitochondrion and the energy production as ATP (see section 2.1.1.2.3).

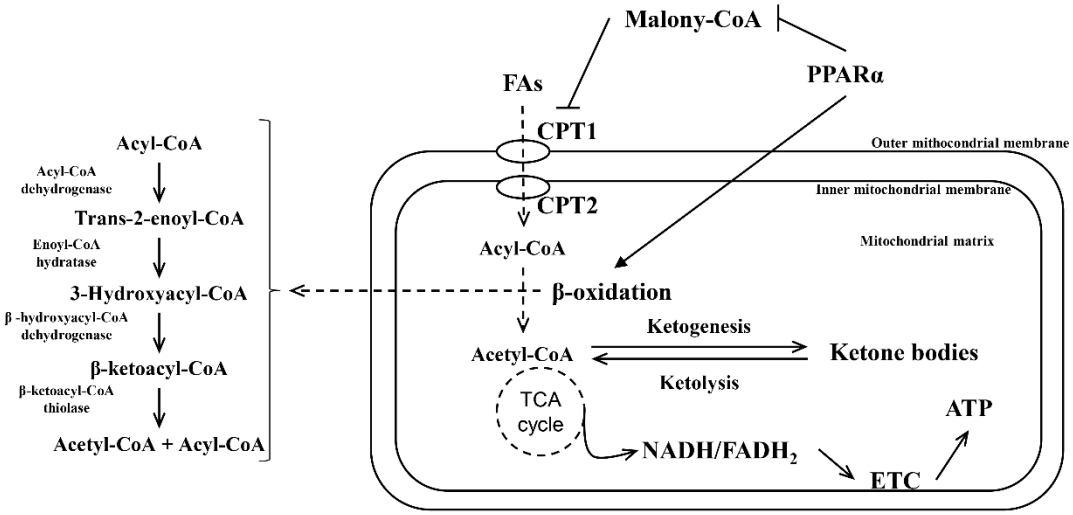

Figure 2.4 Mitochondrial β-oxidation. FAs are degraded through β-oxidation in the mitochondria. First, FAs are transformed to acyl-CoA to be internalized into the mitochondria, where they undergo a series of sequential oxidation reactions to produce acetyl-CoA. This acetyl-CoA can be directed to the TCA cycle to produce energy and reduction power or used to produce ketone bodies (ketogenesis).

The importance of FAO oxidation in NAFLD is not well described, since NAFLD studies in patients have reported both downregulation and upregulation of mitochondrial β-oxidation (Fabbrini et al., 2010; Sanyal et al., 2001; Satapati et al., 2012). It has been proposed that β-oxidation may be increased NAFLD initiation to compensate increased fat accumulation in the liver, however during the preogression of the disease, mitochondrial failure may affect the β-oxidation capacity of the cell. Despite the controversy of these studies, decreased mitochondrial function is considered a common event in NAFLD, in part due to the fact that mitochondrial abnormalities in structure and function are frequently found in NAFLD (Caldwell et al., 1999; Sanyal et al., 2001).

2.1.1.2 Progression to NASH

As mentioned before, simple steatosis can progress to steatohepatitis when a second "hit" is given in the liver, resulting in a damaging situation. Different agents in the liver can lead to the progression of the disease, being the primary event and the most common one the presence of ROS; although there are other important factors, as lipotoxicity and ER stress and mitochondrial dysfunction. The mechanism implicated in NASH progression will be discussed in the next section:

2.1.1.2.1 Reactive Oxygen Species (ROS)

ROS are chemically reactive compounds that are normally generated in the liver and other tissues as a consequence of cellular metabolism of oxygen. ROS have an

important role on cell signalling and can mediate many reactions in the cell, affecting lipids, proteins and DNA (Freeman and Crapo, 1982). There are different ROS components such as hydrogen peroxide (H2O2), superoxide (O2-), hydroxyl radical (OH) and single oxygen (Thannickal and Fanburg, 2000). Under normal conditions, ROS produced in the cells are buffered by the antioxidant machinery (mainly superoxide dismutase (SOD), catalases and glutathione (GSH); however, when the production of ROS overcomes the capacity of its detoxification a situation of oxidative stress is produced in the cell, becoming cytotoxic.

Cellular ROS can come from different sources, mainly mitochondria, endoplasmic reticulum and peroxisomes (Sanyal, 2005). Mitochondria represents the most important producer of ROS: when FAO and TCA cycle are linked to electron transport chain (ETC), a series of reaction of oxidative phosphorylation (OXPHOS) are produced to form ATP. During OXPHOS, e^- and H^+ are transported through the different components of the ETC (each one with higher reduction capacity than the previous one) with the last e^- directed to reduce O2, finally reduced to water in normal conditions. However, during ETC, it is estimated that about 1-2% of the e^- can leak the ETC leading to superoxide radical formation(Boveris and Chance, 1973).

In the pathogenesis of NAFLD, different sources are implicated in ROS overload: increased CYP2E1 expression (a ROS producing enzyme located in the ER and the mitochondria) (Zangar et al., 2004), increased peroxisomal FAO, implicated in H_2O_2 production (Begriche et al., 2006) and, importantly, an excessive flow of e^- derived to the ETC due to increased mitochondrial FAO during NAFLD initiation. The increase in ROS production may induce tumor necrosis factor TNF signalling, that enhances lipid peroxidation, which, in turn, results in increase e^- overproduction, mitochondrial dysfunction and ROS sustained production (Nassir and Ibdah, 2014; Pessayre et al., 2002)

2.1.1.2.2 Lipotoxicity and Endoplasmic Reticulum Stress and inflammation

In the recent years, hepatic lipotoxicity has been regarded as an important contributor to NASH development (Cusi, 2012; Neuschwander-Tetri, 2010). Despite the fact that TG accumulation is the first common step produced in NAFLD, most of the recent studies indicates that TG accumulation itself is not toxic in the liver (McClain et al., 2007). However, besides the amount of FAs, the toxicity in the liver is determined by the relative amount of FA species: while monounsaturated FA (MUFA) do not induced toxicity, saturated FA (SFA) they do (Alkhouri et al., 2009; Listenberger et al., 2003). On

the other hand, several studies have described a role of FFA in the induction of hepatocyte lipoapoptosis through the upregulation of death receptors, such as FAS, TRAIL and DR5, leading to initiation of the extrinsic apoptotic pathway (Feldstein et al., 2003a; Malhi et al., 2007). Upregulation of death receptors is an important feature in liver from NASH patients (Alkhouri et al., 2009; Feldstein et al., 2003b).

In the recent years ER stress has been proposed as an important mechanism implicated in the development and progression of NASH (Malhi and Kaufman, 2011; Ozcan et al., 2004; Puri et al., 2008). By one side, steatosis leads to ER stress as a consequence of FAs and very long chain fatty acid (VLCFA) accumulation and, at the same time, ER stress response contributes to liver damage and NASH progression. ER stress related signalling is linked to lipotoxicity, insulin resistance, inflammation and hepatocyte cell death. ER stress was first described in mouse models of NAFLD has been described (Ozcan et al., 2004; Rahman et al., 2007) and later characterized in NAFLD and NASH human patients (Gregor et al., 2009; Puri et al., 2008). First, during steatosis, ER stress response is implicated in increased insulin resistance and lipogenesis while impairs VLDL secretion contributing to lipid accumulation (Dara et al., 2011; Zhang et al., 2014). During the progression of NASH, ER stress is strongly associated to inflammation by different mechanism (ROS production, activation of NF-κB, JNK and ChREBP transcription factor signalling) and hepatocyte apoptosis (mainly via CHOP induction and JNK/TRAF signalling) (Dara et al., 2011; Zhang et al., 2014).

2.1.1.2.3 Mitochondrial dysfunction

During the development of NAFLD many metabolic adaptations are necessary to counteract the increase of fat in the liver. Mitochondria, as the most important metabolic organelles within the cell, show increased FAO during the initial steps of NAFLD, however, this can lead increased ROS that ends up in mitochondrial dysfunction and ETC deficiency, contributing to the development of NASH. In these sections, a brief introduction of the main metabolic function of the mitochondrion in the liver and its dysfunction in NAFLD will be presented.

a) Mitochondrial role in metabolism

Mitochondria are the main source of energy in hepatocytes and most cells. These organelles are the responsible of generating energy as ATP and reduction power as

NADH and $FADH_2$ through the metabolism of nutrients implicating three converging different pathways: β-oxidation and ketogenesis, TCA cycle and ETC.

TCA cycle

The TCA cycle is the central pathway of metabolism, linking carbohydrate, lipid and protein metabolism through the catabolism of acetyl-CoA. TCA cycle consists of 8 oxidative steps in which acetyl-CoA is oxidized to CO_2 producing ATP, NADH and $FADH_2$ that can be subsequently used as reduction power for the oxidative phosphorylation.

B-oxidation and ketogenesis

FAs are mainly catabolized by β-oxidation in the mitochondria. Dietary lipids can be stored as TGs in the adipose tissue or directly metabolized, depending on the metabolic state. Under certain circumstances such as fasting, TGs stored in the adipose tissue are mobilized to the liver and metabolized for energy production. Once in the hepatocytes, FAs must be activated into acyl-CoA and translocated to the mitochondria, where they undergo cycles, each of four sequential reactions until the FAs are converted into several acetyl-CoA molecules and, in case of impair FAs into acetyl-CoAs and propionil-CoA. Acetyl-CoA at this point can either enter the TCA cycle to produce ATP or be condensated to synthesize ketone bodies (KBs), which are generally oxidized in extrahepatic tissues (Begriche et al., 2013). In the reaction implicated in acyl-CoA catabolism to acetyl-CoA NADH and $FADH_2$ are produced, directly linking mitochondrial FAO with ETC (Figure 2.4).

ETC

As already mentioned in this section, both TCA and FAO converge into the ETC through the production of reduction power NADH and $FADH_2$. These molecules are reoxidized during the oxidative phosphorylation (OXPHOS) reactions that take place in the electron transport chain. OXPHOS are the final step of metabolism, producing ATP in a series of steps controlled by the different components of the ETC: the complexes I, II, II, IV and V (known as ATP synthase) (Berg et al., 2002; Lodish et al., 2000) (Figure 2.5).

Complex I: NADH-ubiquinone oxidoreductase is the complex responsible of NADH oxidation to NAD^+. In this process, two e^- are transferred to the ubiquinone (Q) while four H^+ are translocated to the intermembrane space generating the proton gradient.

Complex II: succinate dehydrogenase (SDH) complex is involved both in the ETC and in the TCA cycle. In the ETC, SDH oxidizes $FADH_2$ to FAD. In this complex, two extra e^- are delivered to the ubiquinone (Q), however, in this case, no H^+ protons are pumped to the intermembrane space.

Complex III: ubiquinone-cytochrome-*c* oxidoreductase complex is involved in the reduction of cytochrome *c* oxidizing the ubiquinol to ubiquinone and contributing to the proton gradient releasing four H^+ to the intermembrane space.

Complex IV: cytochrome *c* oxidase complex is linked to Complex III, transferring four e^- from Complex III to oxygen, producing water and pumping four H^+ the intermembrane space.

Complex V: the ATP synthase couple the ETC to the oxidative phosphorylation, using the proton gradient created across the ETC to generate ATP. This complex acts as an inverted pump that redrives the H^+ to the matrix, using the free energy to produce ATP

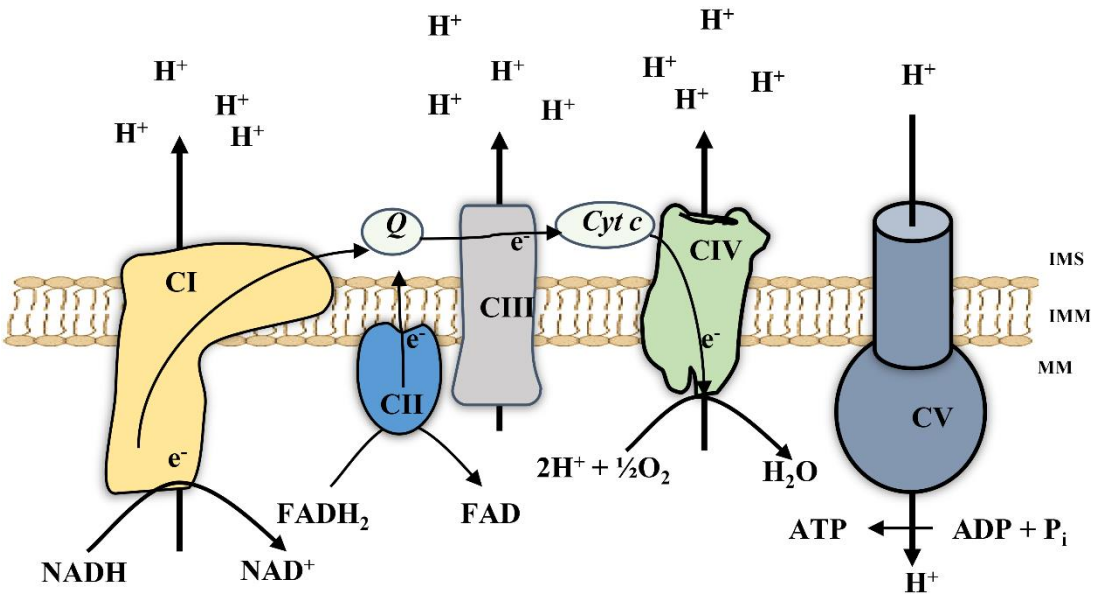

Figure 2.5 Electron Transport Chain. The ETC is composed by a series of complexes that transfer electrons from $FADH_2$ and NADH to the oxygen that is finally reduced to water. During the process, hydrogens are pumped from the mitochondrial matrix to the intermembrane space creating a gradient. Finally, ATP is produced in this process.

b) Mitochondrial dysfunction in NASH

During the last years, increasing studies have pointed NAFLD and NASH progression as a mitochondrial disease. Despite the fact that it is still not clear whether mitochondrial dysfunction observed in NASH is a cause or a consequence of the disease, several studies have clearly shown a link between mitochondrial dysfunction and NASH both in human patients and murine models (Begriche et al., 2013; Nassir and Ibdah, 2014). NASH mitochondrial dysfunctions refer to common events observed such as impairment in ETC complexes activity and reduction in OXPHOS and ATP production. It was first observed in NASH patients the presence of mitochondrial abnormalities (enlarged and swollen and loss of cristae and paracristalline inclusion bodies) (Caldwell et al., 1999; Sanyal et al., 2001). However, in animal model there are some controversies in the way that mitochondrial dysfunctions affect NASH:

Referring FAO, some studies have described increased, unchanged or decreased FAO in different NAFLD murine models (Begriche et al., 2013). However, PPARα reduced expression seems to be a common event in NAFLD, being progressively reduced correlating with NASH progression. Moreover, other studies have described decreased expression of proteins implicated in mitochondrial biogenesis and ETC (PGC1α, NRF1 and Tfam) (Aharoni-Simon et al., 2011; Begriche et al., 2013). In this sense, it seems that mitochondrial FAO is progressively decreased with the severity of NASH.

Similarly, alterations in ETC complexes activity and OXPHOS and ATP production have been described in human patients and murine NASH models. ETC complexes activity was reduced in NASH patients, inversely correlating with plasma TNFα and further decreasing with the higher the progression to fibrosis (García-Ruiz et al., 2013; Pérez-Carreras et al., 2003). Later, similar results showing decreased activity of the different ETC complexes have been reported in NASH murine models (Bruce et al., 2009; García-Ruiz et al., 2006, 2014; Ramirez-Tortosa et al., 2009). These studies highlight the importance of ETC in mitochondrial dysfunction in NASH, potentially linking it with the progressive decrease in energy status and ATP levels in this disease (Cortez-Pinto et al., 1999; Serviddio et al., 2008; Szendroedi et al., 2009).

Potential mechanism implicated in the decrease activity of ETC and FAO during NAFLD include ROS and RNS damaging effect over different complexes; increased inflammatory TNF and interferons signalling implicated in ETC complex impairment,

mtDNA damage and PPARα inhibition; and also lipotoxicity, which can be implicated in ETC enzyme inhibition and even apoptosis (Begriche et al., 2013).

2.1.1.3 NAFLD progression to fibrosis, cirrhosis and HCC

As previously mentioned, the progression of steatosis to NASH and fibrosis and HCC is highly variable and depends on a wide variety of factors, with 10-30% of patients developing NASH, among which 20% progress to cirrhosis and a final 4-27% of the cirrhotic patients ends suffering HCC. Among important factor that influence the grade and time of progression of the disease, there are increasing evidence pointing out the importance of genetic factors that can condition for example the grade of ROS production, the immune system control and the inflammatory response managing the presence and activity of inflammatory cell in the liver, with the concomitant contribution to hepatic stellate cell activation (HSCs) implicated in cell proliferation and extracellular matrix (ECM) deposition. Altogether, factors that can final lead to HCC by different mechanism (Baffy et al., 2012; Calzadilla Bertot and Adams, 2016; Feldstein et al., 2009; Vernon et al., 2011) (Figure 2.1).

2.1.1.4 NAFLD therapies

Despite representing the most common representation of chronic liver disease, to date, there is still no effective treatment approved or effective for NAFLD, being the most frequent recommendation for NAFLD patients focused in changing unhealthy lifestyle (Chalasani et al., 2012; Palmer and Schaffner, 1990; 2002). However, some pharmacological approaches have emerged during the last years. The goal of these pharmacological approaches would be to reduce liver inflammation and injury, overcome insulin resistance and target fibrotic mechanism (Ratziu et al., 2015). Some relevant pharmacological agents are the following:

2.1.1.4.1 Insulin sensitizers

Insulin resistance is present in almost all NASH patients, thus, many pharmacological studies have been focused on the development of insulin sensitizer. Glitazones are the best studied and have the strongest data for the treatment of NASH. Glitazones are compounds that promote differentiation of insulin-resistant large pre-adipocytes into proliferative insulin-sensitive adipocytes thtough the direct activation of PPARγ, enhancing FA uptake in these adipocytes instead of their delivery to the liver. Glitazones also induces insulin sensitivity and adiponectin, increasing FAO in the liver. Moreover, they show some anti-inflammatory effect in Kupffer cells. The best study

glitazone, Pioglitazone, has shown improved individual histological benefits, transaminase (ALT) reduction and correction of insulin resistance in NASH (Ratziu et al., 2015; Sanyal et al., 2010). However, glitazones beneficial effects have resulted short-living after treatment interruption and undesired side effects have been reported (weight gain and bone loss) (Lutchman et al., 2007; Ratziu et al., 2015).

Metformin is an activator of AMP-activated protein kinase (AMPK) that reduces the hepatic glucose production promoting glucose use in peripheral tissues and insulin sensitization. Although it is approved for diabetes 2 treatment, is not recommended for NASH, due to the lack of effectiveness beyond insulin sensitization, it has not shown histological or transaminase beneficial effect in NASH (Chalasani et al., 2012; Zhou et al., 2001).

Finally, other novel insulin sensitizers are being developed as agonist of different metabolic pathways activators. Agonists of the transcription factor FXR (implicated in cholesterol and bile acid metabolism and homeostasis) have shown beneficial effects in insulin sensitization, decreased lipogenesis, increased β-oxidation and reduction of inflammatory processes in murine models, with promising results also in NASH patients (Neuschwander-Tetri et al., 2015; Wagner et al., 2008; Watanabe et al., 2004). Similarly, agonists for the PPARα/δ have been shown to inhibit hepatic lipogenesis and increase fatty acid oxidation, reducing also liver inflammation and fibrosis. PPARα/δ agonist in NASH trials have shown improvement in insulin resistance, dyslipidemia, inflammation and liver function tests (Cariou et al., 2013; Staels et al., 2013).

2.1.1.4.2 Hepatoprotective agents

Antioxidant agents are important to overcome oxidative stress underlying NASH progression in many patients. Vitamin E, protects hepatocytes against mitochondrial toxicity and apoptosis, presenting also antioxidant properties. However, Vitamin E benefit in NASH patients has been only shown in one study and it is not well described whether it may present benefits or not for the treatment of NASH (Hoofnagle et al., 2013; Ratziu et al., 2015; Soden et al., 2007; Sokol et al., 1998).

2.1.1.5 Animal models of NAFLD

The study of NAFLD has been performed in different animal models mimicking the progression of the disease. NAFLD models should reflect the histopathological features presented in NAFLD patients (steatosis, inflammation, hepatocyte ballooning

and fibrosis); however, different models fail to completely address these features. Researchers have used genetic and dietary models to study NAFLD.

2.1.1.5.1 Genetic models of NAFLD

Most important genetic models are based on the absence (Ob/ob) or deficiency (Db/db) of leptin signaling, the hormone that regulates appetite and lipogenesis. Ob/ob mice have a mutation in the leptin gene and develop steatosis, hyperglycemia, hyperinsulinemia, insulin resistance and obesity. However, these mice do not progress spontaneously to steatohepatitis and are resistant to fibrosis development, and, importantly, leptin mutations are not a common feature in NAFLD patients (Takahashi et al., 2012). The Db/db mice have a mutation in the leptin receptor, being resistant to this hormone. These mice are obese and insulin resistant and present hepatic steatosis. However, Db/db mice do not develop steatohepatitis without an extra condition (Wortham et al., 2008). Other important genetic models to study the implication of concrete signalling in steatosis are the Srebp1-c overexpressing mice, PPARα knock out mice, AOX null mice, which differentially develop steatosis through the disruption of the processes in which they are implicated (lipogenesis, FAO and peroxisomal β-oxidation, respectively).

Finally, two important knock-out models are based on the disruption of the methionine cycle and S-adenosylmethionine (SAMe) metabolism, the *Mat1a*$^{-/-}$ (Lu et al., 2001) and the *Gnmt*$^{-/-}$ (Martínez-Chantar et al., 2008). Alterations in this pathway and the enzymes implicated in the cycle have been described in NAFLD patients. Both models are characterized by spontaneous development of steatosis and steatohepatitis at different ages, implicating the disruption of different processes differentially contributing to the development of the disease. *Mat1a*$^{-/-}$ and *Gnmt*$^{-/-}$ and their contributions to NAFLD and liver disease are described in detail in Section 2.2.2 and 2.2.3.

2.1.1.5.2 Dietary models of NAFLD

There are different dietary models extensively used for the study of NAFLD progression: the methyl-choline deficient diet (MCDD), the high-fat diet (HFD), the high cholesterol diet (HCD) and the fructose diet (FD) (Anstee and Goldin, 2006; Hebbard and George, 2011; Takahashi et al., 2012).

MCD diet is characterized by the absence or deficiency of methionine and choline, in the diet, two essential amino acids that are precursors of SAMe and

phosphatidylcholine (PC), respectively. This deficiency compromises important processes such as VDLD formation, methylation reactions and the antioxidant machinery (see section 2.2.1 for detailed functions of methionine and SAMe in the liver). MCDD rapidly induces steatosis, inflammation, cell death, transaminases and fibrosis. Reduced β-oxidation and mitochondrial dysfunctions alongside with increased oxidative stress are characteristic in mice fed the MCD diet. Moreover, MCDD mice is a useful model for the study of NASH specifically in the liver, without other tissue implications. However, these mice differ from human NASH pathology regarding some aspects of the disease, MCDD mice do not gain weight and are not resistant to insulin, while humans NASH are frequently characterized by increased body weight and insulin resistance.

In the HF diet most of the nutrients are derived from dietary fats (71%). HFD induces steatosis, insulin resistance, oxidative stress and inflammation. Therefore, HF is closely related to metabolic syndrome. However, the animals under HFD develop low NAS score, being highly dependent on the mice strain used.

2.1.2 LIVER FIBROSIS AND CIRRHOSIS

As mentioned, NAFLD is a progressive disease that can evolve from the simple steatosis to NASH and fibrosis, being estimated that about 20% of patients suffering NASH finally progress to fibrosis/cirrhosis, a more advanced stage of liver disease (Figure 2.1).

Liver fibrosis is characterized by an excessive extracellular matrix (ECM) deposition in the liver as a result chronic and sustained liver damage with the concomitant sustained wound healing response. The accumulation of ECM proteins alters the normal hepatic architecture of the parenchyma leading to fibrotic scar formation and generation of hepatocyte regeneration nodules, finally leading to cirrhosis. The wound healing response can be initiated in the liver as a reaction to an acute liver damage, but a chronic exposure to the damaging agent is necessary for the fibrosis progression. Damaging agents can be different, including viral infections, autoimmune disorders, alcohol and drug abuse and cholestatic and metabolic diseases. Liver fibrosis can evolve rapidly (weeks or months) in some cases, but normally is a very low progressive disease that take over decades to end up in cirrhosis (Friedman, 2003, 2008a). Cirrhosis is considered as an end-stage of liver disease characterized by distortion of liver parenchyma, nodule formation and hepatic dysfunction or insufficiency, accompanied by decreased

intrahepatic blood flow resulting in portal hypertension (Bataller and Brenner, 2005; Friedman, 2003; Schuppan and Afdhal, 2008).

The architecture of a healthy liver is characterized by a sinusoid surrounded by hepatocytes lined over a membrane of permeable connective tissue called the space of Disse. The inactivated hepatic stellate cells (HSC) reside in the space of Disse while the inflammatory macrophages (Kupffer cells, KC) are located in the sinusoid. During fibrosis, apoptotic hepatocytes activate KCs, which release inflammatory cytokines that activate HSCs. HSCs are the major contributors to fibrosis, once activated secrete large amounts of ECM that fills the space of Disse and remodel the sinusoid, replacing damaged and dead hepatocytes by fibrotic scar tissue. This remodelling of the sinusoid also leads to its capillarization and to the mentioned alteration in hepatic vascularization and portal hypertension, which underlies the main cause leading to cirrhosis complications (e.g. ascites, renal failure, hepatic encephalopathy and varicelar bleeding) correlating with diminished liver functions (Bataller and Brenner, 2005; Schuppan and Afdhal, 2008; Van Beers et al., 2003) (Figure 2.6).

As mentioned, fibrosis can evolve to cirrhosis chronically and, in many cases, asymptomatically. If the liver presents normal or not decreased hepatic functions is known as compensated cirrhosis. Compensated cirrhosis is followed by a progression to decompensated cirrhosis, characterized by the rapid development of different complications associated to hypertension and liver dysfunction that can be accelerated by HCC development and is generally associated to short survival rates.

The diagnosis of cirrhosis still finds its most reliable technique in liver biopsy, it can identify the underlying mechanism of the disease and more accurately set the grade of the progression of cirrhosis. However, it is a very invasive method and it cannot be completely reliable. Alternative diagnostic methods are used, as serum biomarkers and transient elastography (Fibroscan), nevertheless, these new methods present inconveniences as thee do not identify the causes of the disease (Castéra et al., 2005; Pinzani et al., 2005; Schuppan and Afdhal, 2008; Ziol et al., 2005).

Historically, liver fibrosis has been though as an irreversible disease with scar formation being a unidirectional pathway. However, since the late 90's, it has started to be considered as reversible. The most effective therapy for fibrosis is still to eliminate the causative agent underlying the disease (section 2.1.2.3); however, there are

implementation treatments that have shown important improvements in fibrosis regression in patients (Bataller and Brenner, 2005; Benyon and Iredale, 2000; Bonis et al., 2001; Friedman, 2007). The enhancement in the development of effective therapies for liver cirrhosis necessarily pass through the characterization of the major cellular mechanism driving the disease, given the complex interplay of hepatic cells that is known to take place during liver fibrosis.

2.1.2.1 Cell population contribution and fibrogenesis

As mentioned, during liver fibrosis, a complex interplay between different hepatic cellular populations is established (Figure 2.6). In this section, the major contribution of these hepatic cells to liver fibrosis will be presented.

2.1.2.1.1 Hepatocytes

Hepatocytes are the predominant hepatic cells in terms of volume and function. Hepatocytes are generally the most important cells in the initiation of the fibrogenic response. Many damaging and cytotoxic agents (alcohol and drugs metabolites, lipids, bile acids, viruses, etc.) target the hepatocytes, promoting hepatocyte injury. After this initial insult, injured hepatocytes release ROS and cytokines, including inflammatory mediators (interleukins, TNF) and fibrogenic agents (TGFβ), stimulating inflammatory cells recruitment (KCs) and myofibroblast tissue repair activation (HSCs), respectively. Moreover, severe injuries can drive hepatocyte apoptosis, leading to the release of apoptotic bodies from the death hepatocytes. These apoptotic bodies can be phagocyted both by KCs and HSCs, activating them and inducing their production of cytokines, including TNF, TRAIL, FAS-ligand and TGFβ, cytokines that initiate inflammatory and fibrogenic processes in the liver and also increase apoptotic signalling in the hepatocytes (Canbay et al., 2003a, 2004; Higuchi and Gores, 2003; Savill and Fadok, 2000).

2.1.2.1.2 Kupffer cells and immune system

Kupffer cells are the resident macrophages of the liver that are located within the sinusoid and have a high endocytic and phagocytic capacity (including endotoxins and pathogens and apoptotic bodies). These cells are in contact with gut derived and bacterial products that can induce their activity. Upon liver damage, KCs secrete molecules and cytokines (ROS, NOS, TNF, chemokines, etc.) mediating the inflammatory response in the liver and immune system regulation via antigen presentation; and secrete also important death ligands (TRAIL and FAS) enhancing hepatocyte apoptosis. KCs

activation, thus, leads to liver inflammation, hepatocyte apoptosis and also to hepatic stellate cell activation (Canbay et al., 2003a; Gressner et al., 1993).

As Kupffer cells, there are also other members of the immune system described to play a role in liver fibrosis. During hepatic inflammation, innate immune cells (including monocytes, neutrophils, dendritic cells (DC) and natural killer (NK) cells and adaptive immune cells (T and B cells) are recruited to the liver, playing different roles in inflammatory response and fibrogenic development/resolution (Maher, 2001; Winau et al., 2007; Xu et al., 2012).

2.1.2.1.3 Hepatic Stellate Cells

Hepatic stellate cells (HSC; previously known as Ito cells, lipocytes and perisinusoindal cells) are the main contributors to liver fibrosis independently of its etiology, being the major producers of ECM and the amplification of the fibrogenic response (Bataller and Brenner, 2005; Friedman, 2008a; Mederacke et al., 2013). In normal healthy liver, HSCs reside in the space of Disse in contact with the hepatocytes and upon liver injury HSCs get activated and differentiated into myofibroblast-like cells characterized by proliferation, contraction, inflammatory and fibrogenic capacity. Activated HSCs migrate across the liver and accumulate in damaged sites, replacing injured and dead hepatocytes and secreting ECM. HSCs contribution to fibrosis is defined in three sequential steps: *initiation, perpetuation and resolution*.

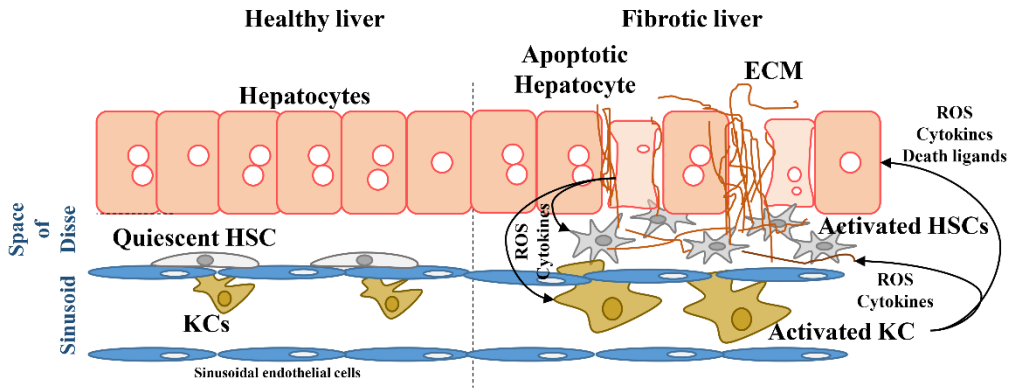

Figure 2.6 Liver architecture and fibrosis. Healthy liver (left panel) is composed by hepatocytes lined on a loose basal membrane (space of Disse) surrounding the sinusoid. In the space of Disse also are found the hepatic stellate cells (HSC) in a quiescent state. Finally, Kupffer cells (KC) are located in the sinusoid. Upon liver injury, fibrosis is initiated in the liver (right panel). Hepatocyte become apoptotic, releasing cytokines that activate both KCs and HSCs. Activated HSCs produce extracellular matrix proteins (ECM) to replace dead hepatocyte and repair tissue. Sustained liver damage, perpetuates interplay between hepatocytes, KCs and HSCs, leading to ECM excessive deposition and parenchymal architecture disruption.

a) Initiation

The initiation phase consists in early and rapid changes in HSCs phenotype and in ECM composition. HSCs become activated very rapidly in response to ROS and cytokines mainly derived from injured hepatocytes, KCs and cholangiocytes. The most important cytokines known to activate HSCs are TGFβ, PDGF and EGF. Another important mechanism of HSCs activation is the engulfment of apoptotic bodies derived from hepatocytes. Similarly to KCs, HSCs have the ability to recognize and engulf these apoptotic bodies from the hepatocytes and also DNA from dead cells (damage/danger-associated molecular patterns, DAMPs), resulting in their activation and proliferation (Canbay et al., 2003b, 2004; Jiang et al., 2009).

Changes in the ECM in this phase consists in changes in collagen composition (from collagen IV as the major component to collagen I and III), changes in membrane receptors (e.g. integrins) (Shafiei and Rockey, 2006; Yang et al., 2003; Zhang et al., 2006) that drives the ability of HSCs to migrate across the matrix, the actin cytoskeleton promotes migration and contraction (Choi et al., 2006; Yee, 1998) and matrix metalloproteases get activated releasing additional growth factors that increase fibrogenic signalling (Schuppan et al., 2001).

b) Perpetuation

Once activated, the HSCs may respond to cytokines and growth factors that enhance their fibrogenic capability through the maintenance and regulation of its proliferation, chemotaxis, fibrogenesis, contractility, proinflammatory signalling and matrix degradation.

Proliferation. HSCs are able to induce their own proliferation by paracrine and autocrine mechanisms involving mainly PDGF, the most potent mitogen described for HSCs (Pinzani, 2002; Pinzani et al., 1994). PDGF regulates proliferation by activating PI3K and MAPK/ERK pathways. Other mitogens responsible for HSCs proliferation are EGF, VEGF and FGF (Friedman, 2008a; Yoshiji et al., 2003; Yu et al., 2003).

Chemotaxis. Hepatic stellate cells are able to migrate across the matrix to the injured place driven by chemoattractants (Ikeda et al., 1999). Potent chemoattractants for HSCs include PDGF, MCP-1 and CXCR ligands (Bonacchi et al., 2001; Kinnman et al., 2000; Marra et al., 1999). HSCs migration is inhibited by the high levels of adenosine at

the place of injury, regulating their fixation and fibrogenesis in the right site (Hashmi et al., 2007).

Fibrogenesis. The main function of activated HSCs is to produce ECM. Fibrogenesis is mainly regulated by TGFβ autocrine/paracrine signalling. TGFβ signalling is intracellular mediated by the Smad2 and 3 receptors (which activate target gene expression associating to transcription factors and coactivators) and Smad7 (which inhibits TGFβ signalling) (Breitkopf et al., 2006; Inagaki and Okazaki, 2007).

Contractility. During liver fibrosis, HSCs presents features characteristics of smooth muscle-like cells, such as the expression of α smooth muscle actin (αSMA) and myosin filaments that mediate contractile activity in these cells (Rockey et al., 1992; Saab et al., 2002). This contractility is one of the major causes of hepatic portal hypertension.

Proinflammatory signalling. Activated HSCs contributes to liver inflammation releasing different cytokines (e.g. CCLs, CXCLs, MCP-1, CCRs, and TNF) that can activate themselves, hepatocytes and other immune cells. Moreover, HSCs are able to interact with immune cells and modulate their response through antigen presentation (Bomble et al., 2010; Friedman, 2008b; Hellerbrand et al., 1996; Lee and Friedman, 2011; Sahin et al., 2010; Wasmuth et al., 2010). Thus, HSCs have the ability to amplify and establish a positive loop of inflammatory signalling that contributes to liver fibrosis.

Matrix degradation. During fibrogenesis matrix remodelling is an important event where HSCs play a major role. In early stage of fibrosis, HSCs release matrix-metalloproteinases (MMPs) MMP-2 and MMP-9 that degrades collagen IV specifically, leading to disruption of the basal membrane in the liver (Arthur et al., 1992; Han et al., 2007). HSCs also release inhibitors of MMPs, TIMPs, important during advance fibrosis as they mainly inhibit metalloproteinases implicated in collagen I and III degradation (e.g. MMP-1), contributing to the perpetuation of liver fibrosis (Benyon et al., 1996; Iredale et al., 1992). Particularly, TIMP-1 is known to inhibit MMP-1 and to have survival effect in HSCs (Murphy et al., 2002). The modulation of TIMPs and MMPs activities is and attractive target to study the reversal of liver fibrosis.

c) Resolution.

During resolution of fibrosis, the excessive ECM deposited in the liver is removed and liver recovers its normal architecture and function. The resolution process requires

HSCs to stop their fibrogenic activity, which occur when HSCs become senescent, inactive or apoptotic (Tacke and Trautwein, 2015). During the resolution there are common events frequently found, such as, decreased production of TIMPs (allowing increase in ECM degradation and collagenase activity (Henderson and Iredale, 2007; Iredale et al., 1998)) changes in the immune system (leading to apoptosis of HSCs, mainly mediated by NKs (Fasbender et al., 2016; Gao et al., 2007; Radaeva et al., 2006).

2.1.2.1.4 Non-hepatic stellate cells

Despite the evidence identifying HSCs as the major contributors to liver fibrosis independently of its etiology (Mederacke et al., 2013), the use of different animal models has allowed to identify other important contributors for the initiation and perpetuation of the fibrotic process. Other sources of myofibroblast in the liver are portal fibroblast (Beaussier et al., 2007; Dranoff and Wells, 2010; Hinz et al., 2007; Iwaisako et al., 2012), bone marrow derived mesenchymal cells (Forbes et al., 2004; Russo et al., 2006) and cells undergoing epithelial-mesenchymal transition (EMT) (Xia et al., 2006) (however, the contribution of the last ones remains controversial (Chu et al., 2011; Scholten et al., 2010; Taura et al., 2010). Particularly, portal fibroblast and epithelial-mesenchymal cells contribution to myofibroblast pool is of special interest in the development of cholestatic disease (an increasing leading cause of cirrhosis beyond alcohol abuse and viral hepatitis B and C) described in detail in the following section (Poupon et al., 2000).

2.1.2.2 Cholestatic liver disease

Cholestatic liver diseases include a wide variety of heterogeneous disorders characterized by the defective bile acid flow from the liver to the intestine, leading to the accumulation of hydrophobic bile acids (BAs) in the liver. This BA accumulation causes initial damage in biliary epithelial cells (named cholangiocytes) and in hepatocytes that can conclude in liver inflammation, liver failure and cirrhosis. Cholestasis can derive either from an impairment in the bile formation in the hepatocytes or from a defective mechanism of secretion at the bile duct (Trauner and Boyer, 2003; Trauner et al., 1998; Zollner and Trauner, 2008). The more frequently causes leading to cholestasis are inflammation, viral infection, drug, hormones, pregnancy and genetic and autoimmune disorders. In adults, cholestatic disorders are more frequently found as chronic primary biliary cholangitis (PBC) or as primary sclerosis cholangitis (PSC); while in children biliary atresia (BA) and Alagille syndrome (ALS) are the most common forms of cholestasis (Bassett and Murray, 2008; Boonstra et al., 2012; Carey et al., 2015; Poupon

et al., 2000; Turnpenny and Ellard, 2012). Cholestatic liver disorders related mortality is not very high, however are relatively common and present lack of effective treatment (surgery being the most frequent option) and frequent evolution to more complicated liver situations (2017). Better understanding of cholestatic liver disease driving mechanisms is necessary to improve therapies and avoid complications

2.1.2.2.1 Pathways implicated in cholestasis

Cholestasis can result from different causes, however, once established, the mechanism and progression underlying this disease are frequently similar, including, alteration in hepatobiliary synthesis, metabolism and transport, biliary epithelial cells damage, hepatocyte apoptosis and inflammation and fibrosis progression, all as a consequence of toxic BA accumulation in the liver. These general features present in cholestasis will be described in this section:

2.1.2.2.2 Bile acids regulation in the liver

Bile acids are the most abundant biliary component and have an important role as regulators of bile flow and the absorption of lipids and other nutrients in the intestine. BAs are generated in the liver as primary BAs (cholic acid, CA and chenodeoxycholic acid, CDCA) through the oxidation of cholesterol and conjugated to glycine or taurine amino acids to form bile salts that can be exported to the intestine through the bile ducts. Once in the intestine, conjugated BAs are partially dehydroxilated and processed by intestinal bacteria, generating secondary BAs (deoxycholic acid, DCA and lithocholic acid, LCA, from cholic acid and chenodeoxycholic acid, respectively) that can return to the liver through the enterohepatic circulation (Chiang, 2013; Russell, 2003).

Alterations in BAs synthesis, export and metabolism drive excessive BAs accumulation in the liver, becoming toxic BAs. Bile acids mainly cause hepatocyte toxicity, but also have strong effect on the biliary epithelial cells (cholangiocytes). In general terms, BAs grade of toxicity correlates with hydrophobicity, the more hydrophobic the BA, the more toxic. Most toxic BAs described are: LTA, DCA, CDCA, GCDCA and TCDCA (Attili et al., 1986; Chiang, 2013; Delzenne et al., 1992).

a) Regulation of bile acid synthesis, metabolism and transport

As just mentioned, BAs are synthetized in the liver as primary BAs. The synthesis of BAs is produced as part of the cholesterol metabolism and is mainly regulated by the enzyme CYP7A1 (a rate limiting enzyme for BAs synthesis) through a negative feedback

mechanism regulated by the BAs pool in the liver. Low BAs returned to the liver induce CYP7A1 activity while, high BA levels inhibit its enzymatic activity (Chiang, 2013). BAs have been described to act as natural ligands of nuclear receptor, regulating its own metabolism. In the process of BA regulation the nuclear receptor transcription factor FXR/NR1H4 play a central through the regulation of the expression of different target genes implicated in BA synthesis, secretion and absorption (Eloranta and Kullak-Ublick, 2008; Makishima et al., 1999; Parks et al., 1999; Wang et al., 1999) (Figure 2.7).

FXR is activated in the liver by different BAs (CDCA, LCA, CA and DCA) and regulates different target genes. One of the main regulatory mechanisms mediated by FXR is the activation of the small heterodimer partner (SHP) that inhibits CYP7A1 activity, thus impeding BA synthesis. Other nuclear receptors are also implicated in the regulation of BA synthesis through the repression of CYP7A1, such as PXR and VDR.

FXR is also implicated in the regulation of BA transporters. At the transcriptional level FXR induces the expression of the bile salt exporting pump (BSEP/ABCB11), the most important exporter of bile acids; the multidrug resistance protein (MDR2), responsible for phosphatidylcholine secretion into the bile and ABCG5/8, implicated in cholesterol secretion. In the intestine, FXR induces the transcription of the organic solute transporter α/β (OST α/β), responsible of the BAs secretion to the blood. Finally, in the hepatocytes FXR is responsible of the inhibition of the NTCP, which mediates BA uptake in the hepatocytes from the blood. Thus, FXR appears as a master regulatory transcription factor that senses BAs levels to regulate its liver synthesis and secretion, intestinal reabsorption and secretion and BA uptake into the hepatocytes (Chiang, 2013; Zollner and Trauner, 2008).

The importance of FXR in cholestasis is further highlighted in studies describing mutations in its gene affecting either the levels or the transcriptional functionality of FXR (with the consequent reduction of its target genes) in human cholestatic diseases such as intrahepatic cholestasis of pregnancy and cholesterol cholestasis (Kovacs et al., 2008; Van Mil et al., 2007). Additionally, in other hereditary diseases, such as progressive familiar intrahepatic cholestasis (PFIC), mutations and defects in BA transporters have been described (Zollner and Trauner, 2008). Nevertheless, such mutational defects are barely frequent in general population and the regulation in BA synthesis and transporters is more frequently found downregulated and upregulated, respectively, indicating

adaptive mechanism to counteract the toxic accumulation of BAs (although not sufficient in most of the cases) (Zollner and Trauner, 2008).

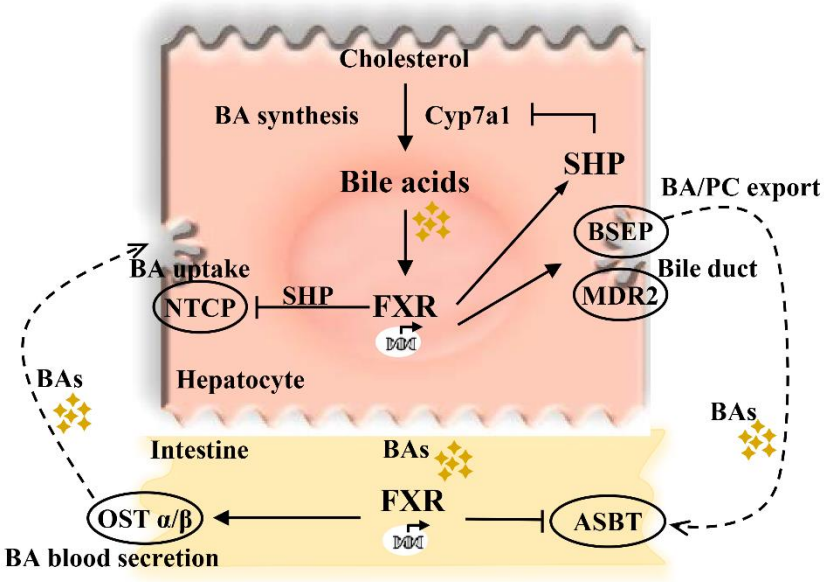

Figure 2.7 FXR and BA regulation. In the hepatocytes, BAs activates FXR transcriptional activity, which activates a set of genes implicated in BA transport, metabolism and synthesis. FXR inhibits BA synthesis through the repression of Cyp7a1. FXR induce the expression of BA exporters (BSEP and MDR2), facilitating the removal of BAs from the liver to the circulation through the bile ducts. In the intestine BA absorption and secretion is also regulated by FXR. Finally, BAs reabsorption from the circulation in the hepatocytes is allowed or impeded by FXR depending on the internal hepatic BA levels. Thus, FXR is a master regulator of BA content in the liver, which functions sensing BA levels.

2.1.2.2.3 Biliary epithelial cell, cholangiocytes

Cholangiocytes are the cells lining the intrahepatic biliary tree that play an important role in bile duct formation and bile acid metabolism and detoxification (Alpini et al., 1988, 1989, 1996; LeSage et al., 1999). During cholangiopathies (e.g. PBC, PSCS), cholangiocytes represent the primary target cell, showing both increase proliferation and apoptosis due to toxicity of BAs. BAs effect in cholangiocytes is highly dependent on the apical bile acid transporter (ABAT) in these cells (Lazaridis et al., 1997), which mediates BAs uptake by the cells with the consequent effect of BAs in cholangiocyte proliferation and BAs secretion (Alpini et al., 1999).

Cholangiocyte proliferation is one of the most important feature in cholestasis and animal models of cholestatic fibrosis (Alpini et al., 1988; Marucci et al., 1993; Roberts et al., 1997) and it does not only depend on bile acids but also on other factors such as intestinal hormones, estrogens, inflammatory cytokines, growth factors and cAMP (LeSage et al., 2001). Resulting cholangiocyte proliferation and malfunction in

cholestasis leads to activation of portal fibroblast and fibrosis initiation, probably by the enhanced cytokine secretion of "activated" cholangiocytes and the recruitment of immune cells (Desmoulière et al., 1997; LeSage et al., 2001; Tuchweber et al., 1996). Several studies have shown the important contribution of cholangiocytes to liver fibrosis, initiating the transformation of portal fibroblast to myofibroblast and recruiting HSCs through the production of several cytokines, such as PDGF (Grappone et al., 1999; Kinnman et al., 2000, 2003), MPC-1 (Lamireau et al., 2003; Marra et al., 1998), TGFβ (Lamireau et al., 1999; Milani et al., 1991) and CTGF (Sedlaczek et al., 2001).

2.1.2.2.4 BA-induced hepatocyte apoptosis

Hepatocytes are the most abundant cells in the liver and the primary target cells in cholestasis alongside cholangiocytes. The accumulation of excessive bile acids in the hepatocytes becomes toxic, resulting in BA-induced apoptosis and necrosis. Different mechanisms have been proposed to contrite to apoptosis in hepatocytes, including FAS and TRAIL-R2 (DR5) mechanism, ROS induction, TNF signalling and ER stress (Figure 2.8).

BAs have been shown to initiate FAS induced apoptosis both in a FAS-ligand independent and dependent manner. FAS receptor can be directly activated by BAs mediating its aggregation on the plasma membrane. Following FAS activation, the caspase cascade is initiated by Caspase 8 and continued by the protease Cathespin B amplifying the apoptotic signalling (Faubion et al., 1999). Similarly, BAs induce apoptosis by induction and aggregation of the TRAIL-receptor2/DR5, leading to the activation of caspase 8 in a Fas-independent way (Higuchi et al., 2001, 2004). These death receptor mechanisms of apoptosis is followed by cleavage and mitochondrial translocation of the proapoptotic protein Bid, which alters mitochondrial membrane potential, leading to depolarization and release of cytochrome *c* to the cytosol, activating the caspase signalling cascade and leading to irreversible cell death (Yin and Ding, 2003).

Another mechanism of BA-induced apoptosis is mediated by ROS. BAs have been demonstrated to induce ROS production in the mitochondria (Sokol et al., 1993, 1995). ROS induction its implicated in the activation of JNK and the protein kinase C, which finally phosphorylates FAS, inducing its localization into the plasma membrane (Sodeman et al., 2000). Parallel, PKC have been proposed to induce cathespin activation and Mg^{2+} entrance in the cell with the following endonuclease activity and DNA cleavage (Patel et al., 1994). On the other hand, ROS also induces the intrinsic mitochondrial

pathway of apoptosis. BA accumulation increases mitochondrial ROS and leads to mitochondrial membrane depolarization and release of proapoptotic proteins such as Cyt *c* and the caspase cascade initiation. The direct implication of ROS and membrane depolarization in BA-induced apoptosis has been further demonstrated by the reduction of apoptosis when using ROS and membrane depolarization inhibitors (Botla et al., 1995; Yerushalmi et al., 2001).

Finally, another mechanism implicated in bile acid-induced apoptosis has been recently described. BAs mediates upregulation of CHOP, which is an important mediator of the ER stress induced apoptosis, leading to hepatocyte cell death(Tamaki et al., 2008).

In summary, hepatocyte apoptosis is a well-documented event occurring during cholestasis (*in vitro* and *in vivo*) playing an important role in the progression of the disease, contributing to liver dysfunction and increasing the inflammatory and fibrogenic processes associated to BA accumulation in the liver.

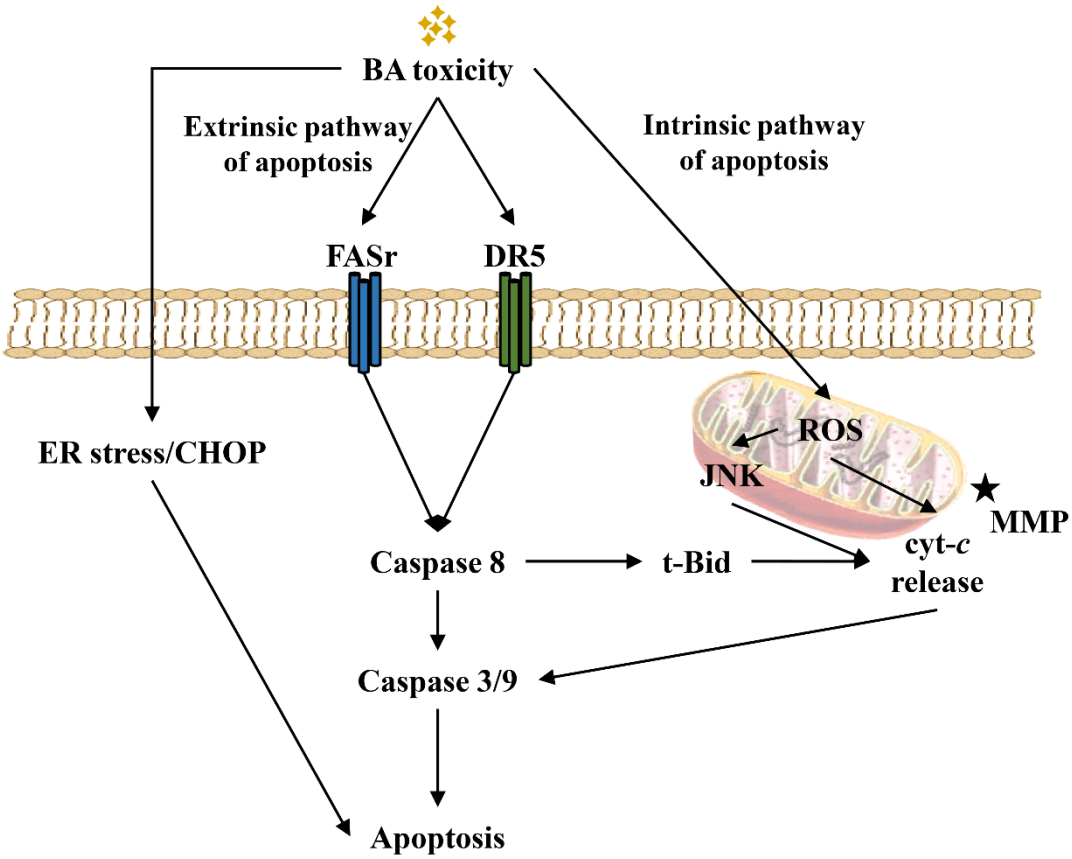

Figure 2.9 Pathways implicated in BA-induced apoptosis. BA accumulation in the liver become toxic and induced apoptosis by the extrinsic and intrinsic pathways. BAs engage FASr and TRAIL-receptor2 (DR5) activating caspase cascade. BAs also induce mitochondrial ROS, triggering JNK activation, loss of mitochondrial membrane potential (MMP), cyt-*c* release and apoptosis.

2.1.2.3 Liver fibrosis therapies

Nowadays, there is no effective and standard treatment for the intervention of liver fibrosis. Most effective current treatment implicates the removal of the causative agents underlying the disease. The recent success achieved in blocking and reversing the progression of liver fibrosis with antiviral treatments has opened the possibility of treatment liver fibrosis. However, considering the high prevalence of fibrosis progression from NASH patients, there are still some major challenges for the development of new therapeutical approaches: I) better characterization of the disease and molecular pathways underlying; II) non-invasive markers for the diagnostic of the disease and III) establishment of continued studies for the progression of the disease in treated patients.

Some of the targets of current research in fibrosis treatment include: anti-inflammatory drugs to avoid inflammation contribution to progression of the disease; targeted therapy against HSCs to inactive them or to induce their apoptosis; antioxidants therapies to protect the hepatocytes from ROS induced damage; synthetic transcription factor ligands (PPARs and FXR); and the use of the non-toxic ursodeoxycholic bile acid (UDCA) for the treatment of BA-induced fibrosis (Bataller and Brenner, 2005; Trautwein et al., 2015).

2.1.2.4 Animal models of fibrosis

Animal models have been extensively used in fibrosis research, greatly contributing to the understanding of the disease. There are several animal models of liver fibrosis, however, all of them present different characteristics that needs to be accounted, contributing unequally to the disease (genetic background, immune system contribution, differential gene expression, etc.) Some of the most animal models used for the study of liver fibrosis are based on chemical toxins, such as carbon tetrachloride (CCl_4); surgical procedures, the bile duct ligation (BDL); diet models, the MCDD; and some genetic models (*Md2*$^{-/-}$ and *Gnmt*$^{-/-}$).

Carbon tetrachloride (CCl_4) is a chemically induced model of fibrosis. CCl_4 is intraperitoneally administrated and transformed into CCL_3^- by CYP2E1in the liver. CCL_3^- radical leads to an acute phase of hepatocyte cell death, necrosis, inflammation and activation of fibrogenesis. Sustained administration leads to fibrogenesis and even HCC development (Scholten et al., 2015). Other chemical-based models of fibrogenesis use ethanol, DMN and DEN compounds, which are also CYP2E1 related toxins (Starkel and Leclercq, 2011; Yanguas et al., 2016).

Bile duct ligation (BDL) is a surgical procedure that involves the ligation of the bile duct, leading to obstructive cholestasis. The ligation of the bile duct leads to bile acid accumulation in the liver, which cannot be secreted. The excess of bile acids promotes hepatocyte apoptosis, inflammation and fibrogenesis. BDL model is characterized by the implication of portal myofibroblast in the fibrogenic response as well as by the proliferation of the cholangiocytes and the intrahepatic bile ducts. These characteristics make BDL an excellent model frequently used to study biliary cirrhosis. The invasiveness and difficulty of the procedure and the high mortality associated to the process are the major disadvantages of BDL model (Tag et al., 2015).

Animal models of diet induced fibrosis includes the MCDD. As mentioned in section 2.1.1.5.2, this diet induces the progression of steatosis to NASH and fibrosis by disrupting the methionine cycle and the glutathione synthesis and increasing oxidative damage. Another important diet that induces cholestatic fibrosis is a high content bile acid diet, which promotes toxic bile acid accumulation and cholangiocyte proliferation, resembling the BDL mouse model.

Finally, genetic models are also important in the study of liver fibrosis. In this sense, the most important is the *Mdr2*$^{-/-}$ mouse (Popov et al., 2005). This mouse lacks the Mdr2 protein that is responsible for the secretion of phospholipids into the bile from the liver and spontaneously develops severe biliary fibrosis and HCC. The previously mentioned *Gnmt*$^{-/-}$ mouse has been also used for the study of fibrosis, as these mice spontaneously progress from steatosis to NASH, fibrosis and HCC (section 2.2.3.1). In this mouse, the characteristic chronic excess of SAMe contributes to alterations in the immune system during NASH and fibrosis, leading to overactivation of NK/NKT cells, which promote TRAIL-induced apoptosis in hepatocytes, highlighting the contribution of SAMe metabolism and immune system and TRAIL to liver injury (Fernández-Álvarez et al., 2015; Gomez-Santos et al., 2012).

2.1.3 HEPATOCELLULAR CARCINOMA

2.1.3.1 Epidemiology and etiology

Liver cancer is an important cause and morbidity and mortality over the world. It is the fifth most common cancer and the second most common cause of cancer-related cell death, being HCC the most frequent presentation of liver cancer (70-85%) (Govaere and Roskams, 2015; Jemal et al., 2011). Other liver cancer types are cholangiocarcinoma,

hemangiosarcoma and hepatoblastoma. The etiology of HCC is heterogeneous and multifactorial and the major risk factor for the development of HCC are chronic hepatitis B and C, alcoholism, aflatoxin B1 intoxication and NAFLD (described in more detail in the next section) (McGlynn and London, 2011; Mittal and El-Serag, 2013). HCC is frequently asymptomatic in early stages of the disease being diagnosed at late stages, when it is already presented as multifocal and alongside a cirrhotic surrounding environment, making HCC of difficult treatment and a poor prognosis cancer (Attwa and El-Etreby, 2015; El-Serag et al., 2008; Llovet and Bruix, 2003). At the moment of diagnosis very few patients are eligible for therapeutic intervention (liver transplantation or tumor resection). Survival rates of HCC patients ranges between 6-20 months after diagnosis. Tumor recurrence after therapeutically intervention is frequent and may be enhanced by the convergence of different signalling pathways contributing to the malignant transformation of the HCC, difficulting the efficacy of conventional systemic therapies (Forner et al., 2012). Better understanding of molecular pathways driving hepatocellular carcinoma is needed for the development of new strategies for HCC treatment.

2.1.3.1.1 NAFLD and HCC

As previously mentioned, there is approximately a 4-27% of cirrhotic patients that develops HCC (Figure 2.1). The increase of NAFLD incidence in the last years and its expected continued rise in the next future is positioning NAFLD-associated HCC as one of the second leading causes of HCC and the most increasing one, countering the recent progress achieved in the treatment in HBV/HCC-derived HCC (Khan et al., 2015; Michelotti et al., 2013; Wong et al., 2014). Many risk factors mentioned before for NAFLD development are also risk factors for HCC (metabolic syndrome, dyslipidemia, diabetes, etc.) and almost universally presented at least in one form in NAFLD-derived HCC (Michelotti et al., 2013; Welzel et al., 2011). One important feature of NALFD-derived HCC is the possibility of developing HCC without cirrhosis (Alexander et al., 2013; Ertle et al., 2011; Guzman et al., 2008). Despite these particular features characterizing this type of HCC, the mean survival is similar to other etiologies. Improvement in understanding and diagnosing HCC and NAFLD-derived HCC would help to reduce HCC increased incidence during the last years, with NAFLD rising popping up as the major contributor for this increase in HCC (Khan et al., 2015; Wong et al., 2014).

2.1.3.2 Molecular pathways in HCC

One of the principal features that makes HCC a highly difficult to treat and a very poor prognosis cancer is its particular heterogeneity, with many different molecular signalling pathways activated at the same time and contributing to the development of the cancer. Some important frequent alterations found in HCC are the following:

2.1.3.2.1 Signalling pathways in HCC

HCC is driven by a variety of signalling pathways implicated in the regulation of cell growth and proliferation, differentiation, angiogenesis, inflammation and apoptosis.

a) Tyrosine kinases receptor (TKRs) pathway

TKRs include a group of receptor whose activation involves different growth and migration pathways. Most important pathways responding to TKRs activation are Ras-MAPK (Ras/Raf/MEK/ERK), PI3K/Akt, VEGF, EGFR, c-MET and c-Myc. Ras-MAPK and PI3K/Akt are frequently overactivated in early HCCs and in almost all advanced HCC (Bhat et al., 2013; Calvisi et al., 2006; Schmidt et al., 1997). Ras-MAPK is activated by different TKRs as IGFR, EGFR, PDGFR and FGFR and lead to the activation of transcription factors and proliferation genes. On the other hand, PI3K/Akt is activated by IGFR1 and other TKRs and activates the mTOR signalling pathway. PI3K/Akt pathway is also regulated by inactivation by PTEN, which frequently downregulated in HCC by loss of function, mutations or epigenetic silencing (Figure 2.9).

b) VEGF angiogenic pathway

HCC is a highly vascular tumor in which the formation of new vessel to sustain vascularization is very important. However, HCC, as well as many other solid tumors, is characterized by the presence of hypoxic regions that induces an angiogenic response to generate the growth of new vessel from the surrounding parenchyma into the tumor. The angiogenic necessities in HCC and the response to hypoxic conditions is mainly mediated by the overexpression of VEGF, the major player in tumor vascularization (Kim et al., 1998; Muto et al., 2015).

c) JAK/STAT pathway

JAK/STAT signalling pathway is frequently found overexpressed in HCC and promotes transcription of genes implicated in proliferation, migration and differentiation. JAK/STAT pathway is autoregulated in a negative feedback loop in which JAK/STAT

activation induces the transcription of SOCS proteins, which binds to JAK to inhibit the pathway. The frequent overexpression of JAK/STAT signalling in HCC is associated to the high methylation of different SOCS promoter, preventing the negative autoregulation of the pathways (Calvisi et al., 2006).

d) Epigenetics: DNA methylation and microRNAs

Epigenetics is a term used to define a variety of mechanism implicated in the control of gene expression without affecting genomic sequences. Epigenetic regulatory mechanisms include DNA methylation, histone acetylation/methylation, microRNAs, transcription factors and chromatin remodeling. Alterations in the regulation of this mechanisms are highly involved in HCC development and prognosis. Basically, epigenetic modification contribute to cancer development by enhancing tumor oncogenic gene expression or downregulating tumor suppressor genes. Epigenetics in liver disease and HCC are described in more detail in section 2.3.

e) Other pathways

Other important pathways contributing to HCC are the WNT/β-catenin and the TGFβ pathways. WNT/β-catenin is implicated in the regulation of proliferation associated genes and is frequently activated in HCC mainly responding to different mutations (Thompson and Monga, 2007). TGFβ plays a dual role in HCC development, acting as a tumor suppressor during HCC initiation and being implicated in invasiveness, angiogenesis and metastasis in advanced HCC (Roberts and Wakefield, 2003).

2.1.3.3 HCC treatment, sorafenib and drug resistances

Hepatocellular carcinoma is still considered as a very poor prognosis cancer due to the difficult and low effective therapeutic options still available for its treatment. Despite the recent progress in the treatment of HCC since the approval of sorafenib as the unique drug for HCC systemic treatment, the results in terms of surveillance are still low and need to be improved. Therapeutical options considered for HCC depends on the stage of the disease and are the following:

a) Resection and transplantation

Resection is usually the first considered option for patients with less than three localized tumor without surrounding cirrhosis (Bruix et al., 2015; Yu, 2016). It implicates the removal of the tumoral tissue and the non-tumoral surrounding tissue. Resection rises

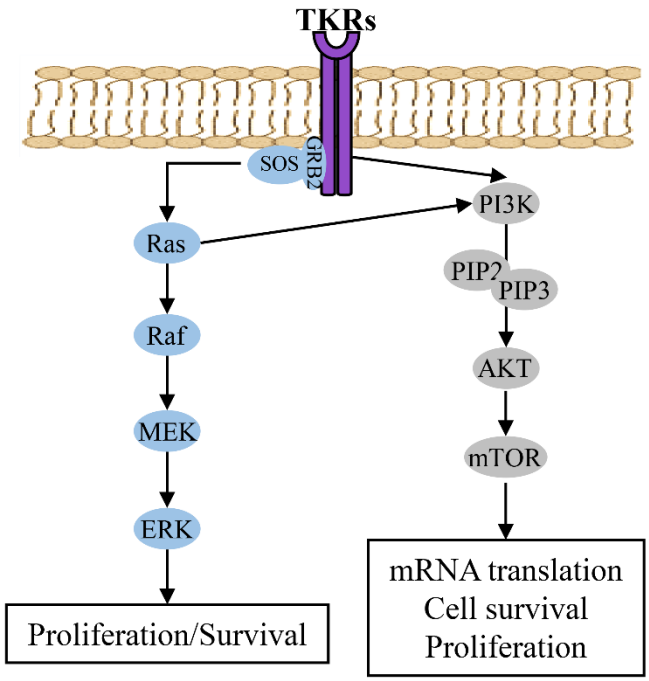

Figure 2.9 TKR signalling in hepatocellular carcinoma. Tyrosine kinase receptors (TKRs) are frequently overactivated in HCC, activitating oncogenic signalling cascades related to different properties of HCC. Ras/raf/MEK/ERK and PI3K signalling pathways are frequently overactivated in HCC by different TKRs, conferring proliferation and survival advantages to hepatocellular carcinoma.

several major problems as it is a highly aggressive intervention and recurrence is frequently observed after 5 years (70%). Complications in HCC patients with cirrhosis are greater, including possibility of liver failure (Bruix et al., 2015).

Liver transplantation is more frequently performed in patients with decompensated cirrhosis, included in the Milan criteria (single nodule <5 cm or <3 nodules, without vascular invasion). The overall survival of patients undergoing liver transplantation is higher than those with resection, and recurrence is significantly lower (Liver and Cancer, 2012; Llovet et al., 1999). The major problem concerning liver transplantation is the low availability of liver donors.

b) Locoregional therapy

These non-surgical therapies are frequently used in combination with surgery or when surgery is not an option in patients presenting intermediate HCC. They include tumor radiofrequency ablation and TACE (transcatheter arterial chemoembolization). Tumor ablation induces tumor necrosis by heat (generated by radiofrequency, laser cryoablation, etc.). TACE is more frequently used in patients with large multifocal HCC

and it is based in the injection of chemotherapy through the arteries followed by ischemia-embolization (Llovet et al., 2002).

c) Systemic therapies

Systemic therapy is normally directed to patients with advanced HCC that are not eligible for other mentioned therapies or to those patients with low response to previous treatments. Nowadays, sorafenib is the only drug approved for the systemic therapy of HCC. Sorafenib is a multikinase inhibitor that acts mainly upon Ras/Raf/MEK/ERK, VEGF and PDGF pathways (Wilhelm et al., 2006). Sorafenib treatment has shown improved survival in advanced HCC patients in initial studies (Llovet et al., 2008), however, loss of efficacy, development of resistances and side effects have evidenced the need of improvements in HCC-sorafenib based therapies (Berasain, 2013). The following section will present the importance of sorafenib in HCC treatment, regarding its molecular mechanism, efficacy and resistances.

2.1.3.3.1 Sorafenib

The advances produced during the last decades in understanding cancer biology and molecular mechanism implicated in oncogenesis and tumor development have led to the increased development of rationally-designed drugs instead of non-specific cytotoxic chemotherapeutic agents for the treatment of cancer. The most important drug recently approved for the treatment of HCC is sorafenib, a multikinase inhibitor that has also shown benefit in other cancer types.

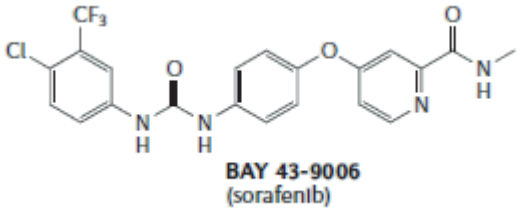

Figure 2.10 Chemical structure of Sorafenib (from Wilhelm et al 2006).

Sorafenib (Nexavar, BAY 43-9006, Bayern Pharmaceuticals) is a multikinase inhibitor with proved antitumoral activity in HCC and other cancer types (Liu et al., 2006; Llovet et al., 2008; Wilhelm et al., 2006, 2004) (Figure 2.10). This multikinase has been shown to inhibit both proliferation and angiogenesis acting through different targets: it inhibits the serine/threonine kinases c-RAF and BRAF, as well as the angiogenic receptor tyrosine kinases VEGFR2/3, PDGFR, ret and c-Kit. As mentioned in section 2.1.3.2.2,

HCC tumors show overactivation of tyrosine/kinases pathways and are highly dependent on blood supply and vascularization, thus the rationale use of sorafenib in HCC emerges as an interesting therapeutical option. As mentioned, sorafenib directly inhibits Ras/Raf/MEK/ERK in HCC through the direct inhibition of Raf kinases, with the consequent ablation of MEK/ERK signalling pathway and cyclin D1 dependent proliferation (Liu et al., 2006). Parallel, sorafenib also inhibits the phosphorylation and autophosphorylation of angiogenic tyrosine/kinase receptors VEGFR and PDGFR, contributing to its antitumoral effect (Liu et al., 2006; Wilhelm et al., 2006). Finally, in HCC, an important role of sorafenib in the inhibition of transcription and proliferation through STAT3 inhibition has been described (Blechacz et al., 2009; Chen et al., 2010; Tai et al., 2011) (Figure 2.11).

Besides antiproliferative and antiangiogenic properties, sorafenib has also been demonstrated to exert proapoptotic effects in HCC (Figure 2.11). Regarding induction of apoptosis, different mechanisms have been proposed to underlie sorafenib induced apoptosis. First, sorafenib was proposed to inhibit MCL-1, an anti-apoptotic Bcl-2 protein member, through the inhibition of phosphorylation of eIF4E in HCC cell lines (Liu et al., 2006). Sorafenib inhibition of MCL-1 has been shown in other cancers such as leukemia (Rahmani et al., 2005; Yu et al., 2005). Second, a role of the p53-upregulated modulator of apoptosis (PUMA) has been proposed to be essential for apoptosis initiation mediated by sorafenib in HCC (Dudgeon et al., 2012). PUMA is a BH3-only Bcl-2 family member that functions as a critical initiator of apoptosis in cancer cells through the inhibition of anti-apoptotic Bcl-2 protein members and activation of pro-apoptotic members, resulting in mitochondrial dysfunction and caspase cascade activation (Yu and Zhang, 2008). PUMA is classically induced by P53 upon DNA damage, however it has been demonstrated that can be induced through p53-independent mechanism (TNF, P73, NF-κB or c-JUN) (Cazanave et al., 2010; Ming et al., 2008; Wang et al., 2009). Regarding PUMA induction by sorafenib, it has been described to occur in a P53-independent manner, through the activation of the NF-κB transcription factor (Dudgeon et al., 2012). Other studies have describe other mechanisms as transcription factors (GADD45β) (Ou et al., 2010) and physiological apoptotic stimulation (e.g. TGFβ and TNF) (Fernando et al., 2012), implicating in some cases activation of JNK/c-JUN signalling pathways as essential for the response to sorafenib (Fernando et al., 2012; Lin et al., 2017; Ou et al., 2010; Wei et al., 2010; Yu et al., 2006). Interestingly, despite the well characterized role

of P53 in induction of apoptosis in HCC and many other cancer cell, sorafenib induced apoptosis have been proved to be P53-independent in several of these studies, affecting sorafenib in the same extent to P53-wild-type/mutant cells.

Recent progress above mentioned in understanding sorafenib mechanism will help to improve sorafenib treatment in HCC patients, especially regarding the loss of effectiveness and appearance of resistance upon the continued treatment with sorafenib. In this context, there have been only one effective pharmacological combined therapy improving survival in HCC patients showing no benefit from sorafenib treatment. Regorafenib, a multikinase inhibitor that blocks the activity of kinases involved in angiogenesis, oncogenesis, metastasis and tumor immunity is approved for the treatment of metastatic colorectal cancer and gastrointestinal tumors, and have been shown beneficial effects in a phase 3 study in HCC patients treated with sorafenib (Bruix et al., 2017).

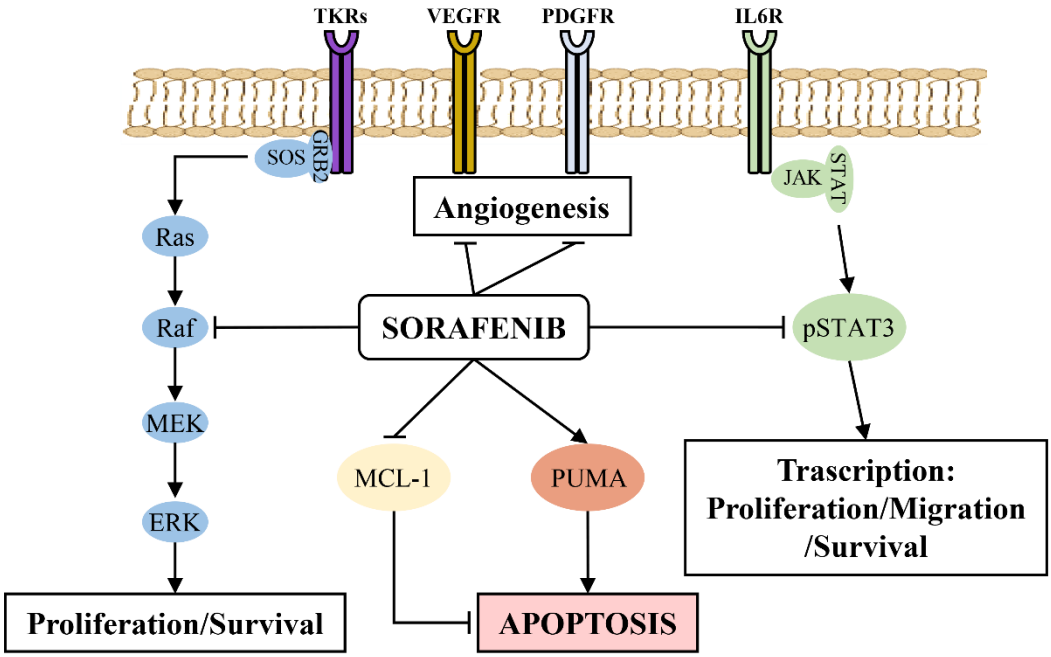

Figure 2.11 Sorafenib mechanisms in HCC. Sorafenib have been proposed to inhibit HCC proliferation by different mechanism. Sorafenib blocks proliferation and survival by inhibiting Raf/ERK signalling. Sorafenib also blocks angiogenesis acting over the TKRs VGFR and PDGFR and inhibits transcription and survival signaling governed by STAT3. Sorafenib directly induces apoptosis inhibiting MCL-1 antiapoptotic protein and activating PUMA effector of apoptosis.

2.1.3.3.2 Drug resistances in HCC

One of the major clinical challenge in the treatment of HCC relies behind overcoming drug resistances frequently presented in HCC patients. It has been shown that

HCC presents several different mechanisms implicated in non-acquired (primary) and acquired resistance to different therapies. This is in part due to the heterogeneity underlying HCC development, related also to the high genetic instability characteristic of HCC and the tumoral response to the therapy. In this sense, different mechanisms of chemoresistance (MOCs) can be divided in five principal groups affecting: changes in the intracellular drug concentration by decrease drug uptake (MOC-1a) or export (MOC-1b), enhanced drug inactivation or decreased prodrug activation (MOC-2), changes in the molecular targets of the drugs (MOC-3), increased repair response to drug-induced DNA damage (MOC-4) and misbalance between signalling mechanisms implicated in survival and apoptosis (Marin and Briz, 2010; Marin et al., 2017). In this thesis, we have studied sorafenib resistances regarding intracellular levels of sorafenib and mechanisms of apoptosis (i.e. MOC-1a-b and MOC-5).

a) Sorafenib uptake/export pumps

MOC-1a-b involves mechanisms implicated in reducing the intracellular levels of the therapeutic drugs, including drug transporters responsible for the drug uptake (MOC-1a, solute carrier family, SLCs) and drug export (MOC1-bexporting pups belonging to the ATP-binding cassette ABC superfamily, MRPs/MDRs) implicated in the development of the multi drug resistant (MDR) phenotype (Marin et al., 2014, 2010). Different transporters included in the MOC-1a have been found downregulated in HCC and other liver cancers, being the most important SLC22A1, implicated in the intracellular uptake of sorafenib as well as other drugs (e.g. platinum based drugs and doxorubicin) (Herraez et al., 2013; Martinez-Becerra et al., 2012; Zollner et al., 2005). On the contrary, some export pumps (e.g. MDR1, MRP2 and MRP4) are frequently overexpressed (in basal levels or response to treatment) or related to poor prognosis in HCC (Marin and Briz, 2010; Ng et al., 2000; Wakamatsu et al., 2007).

a) Mechanism evading sorafenib-reduced viability and apoptosis

There are different mechanisms implicated in loss of drug efficacy. In the case of sorafenib, there are different pathways that have been described to play a role in HCC resistance, such as activation of proliferation compensatory pathways (PI3K/AKT), induction of EMT, activation of hypoxia-related signalling, dysregulation of pro/anti-apoptotic proteins and genetic/epigenetic characteristics of the tumor than can contribute to the different response (Berasain, 2013; Zhai and Sun, 2013). Importantly, regarding

epigenetic characteristics of the tumor, microRNA signature and response to different drugs have emerged in the recent years as important contributors to the development of drug resistance (Fornari et al., 2009, 2017; Xia et al., 2006; Xu et al., 2016).

In summary, different mechanisms are implicated in primary/acquired resistance to sorafenib in HCC. Since sorafenib represents the unique drug for systemic treatment of HCC but has shown only relevant improvement regarding overall survival in patients, uncovering these resistance mechanisms will be highly relevant for the treatment of HCC.

In the next two sections the importance of methionine metabolism in the liver and the role of epigenetics as important drivers of chronic liver disease progression (NAFLD, fibrosis and HCC) will be presented.

2.2 METHIONINE METABOLISM IN LIVER DISEASE

Several studies have linked alterations in methionine metabolism with development of liver disease. First insight into the role of methionine metabolism was in 1932, Best demonstrated that rats fed with a diet deficient in methyl groups (methionine, choline and folates) developed liver steatosis, which progressed to steatohepatitis, fibrosis and HCC when prolonged in time (Best et al., 1932). Later, in human patients suffering cirrhosis, Kinsell demonstrated the deficient methionine clearance from the plasma in those cirrhotic patients, linking liver disease and hypermethionemia (Kinsell et al., 1947, 1948). Since then, the link between methionine metabolism and liver disease have been largely studied, providing important evidence into its regulation by different enzymes and its implication in several pathways differently implicated in the development of liver disease.

2.2.1 METHIONINE, S-ADENOSYLMETHIONINE AND METHIONINE CYCLE

Methionine is an essential amino acid that is converted into S-adenosylmethionine (SAMe, SAM or AdoMet) in a reaction catalyzed by the enzyme methionine adenosyltransferase I and III (MATI/III), using ATP as co-substrate (Cantoni, 1953; Cantoni and Durell, 1957). SAMe is the most important biological methyl donor and it can be produced in all the cells, although the liver is the responsible of approximately the 50% of methionine metabolism and the 85% SAMe methylation reactions (Finkelstein, 1990; Mato et al., 2002; Mudd and Poole, 1975). SAMe is involved in transmethylation reactions, it can donate the methyl group to DNA, RNA, proteins, amino acids, sugars

and phospholipids in reactions catalyzed by specific methyltransferases (Mato et al., 2008, 2013; Petrossian and Clarke, 2011). Moreover, SAMe not only participates in transmethylation reactions but it is also involved in polyamines synthesis and in the transulfuration pathway to generate glutathione, the main antioxidant in the cell (Lieber and Packer, 2002; Lu, 2000; Mato et al., 1997).

Methionine and SAMe levels are controlled by a set of enzymes that participates in the named "Methionine cycle" (Figure 2.12). In this cycle, SAMe is synthetized from L-methionine in an ATP-dependent reaction by MATI/III. Then, SAMe can be demethylated by different methyltransferases, being the most important glycine N-methyltransferase (GNMT, section 2.2.4), generating S-adenosylhomocysteine (SAH), an inhibitor of many methyltransferases. In this sense, GNMT is the responsible of the maintenance of SAMe/SAH ratio, considered as the indicator of the methylation capacity of the cell (Finkelstein, 2007). SAH is hydrolyzed by SAH hydrolase (SAHH), to prevent SAH accumulation, in a reversible reaction that generates homocysteine (HCY) and adenine. Homocysteine can enter two pathways then: the remethylation and the transulfuration pathways. The transulfuration pathway is of particular importance in liver because of its high activity. In this transulfuration pathway, homocysteine is used as a substrate of cystathionine-6-synthase (CBS), generating cysteine and glutathione (Lu, 1999, 2009).

Alternatively, methionine can be regenerated from homocysteine through the remethylation pathway, which can be directed by two different enzymes: betaine homocysteine methyltransferase (BHMT) and methionine synthase (MS). BHMT is a liver and renal specific enzyme, which converts homocysteine into methionine using betaine as co-substrate. MS-mediated remethylation requires normal levels of vitamin B_{12} and folates and it is coupled to the folate cycle. MS uses 5-methyltetrahydrofolate (5-MTHF) as methyl donor for HCY, generating methionine and tetrahydrofolate (THF). THF is then converted to 5,10-MTHF by the enzyme 5,10-MTHF synthetase (MTHFS) and finally regenerated to 5-MTHF by the last enzyme of the cycle, MTHF reductase (MTHFR) (FIG 2.12).

The fate of HCY to enter transulfuration or transmethylation pathways is determined by the hepatic levels of SAMe, which is an activator of CBS and an inhibitor of MS and MTHFR activities. Thus, when SAMe levels are high, HCY enter the

transulfuration pathway, while low SAMe levels directs HCY to the remethylation pathway to regenerate methionine and SAMe (Mato et al., 1997; Prudova et al., 2006).

An alternative pathway of the methionine pathway is the use of SAMe for polyamine synthesis. In these pathways, SAMe is not metabolized by any methyltransferase but is decarboxylated by a specific SAMe decarboxylase in a reaction that generates two molecules of 5'-methylthioadenosine (MTA). MTA is an inhibitor of polyamine synthesis (Pegg and Williams-Ashman, 1969), methylation reactions (Dante et al., 1983) and SAHH activity (Della Ragione and Pegg, 1983) and can affect gene expression, proliferation, differentiation and apoptosis (Avila et al., 2004). MTA is removed to restore methionine levels in a process known as the methionine salvage pathways (Avila et al., 2004) (Figure 2.12).

Methionine and SAMe levels and metabolism are frequently found altered in liver disease. The regulation of the methionine cycle is controlled, as we have mention previously, by enzymes and by the products generated during different reactions over the cycle. For example, SAH is a potent inhibitor of SAMe-mediated methylation reactions and high levels of SAMe activate CBS and transulfuration pathway while inhibiting MTHFR and regeneration pathway. The alteration in the expression of the enzymes implicated in methionine and SAMe metabolism leads to whole dysregulation of the methionine cycle. Low levels of SAMe are frequently found in liver disease, as a consequence of low expression of *MAT1A* gene and MATI/III enzymes (Avila et al., 2000; Duce et al., 1988). However, on the contrary, the gene codifying for the enzyme implicated in SAMe catabolism, *GNMT*, is also found downregulated in liver disease, contributing to abnormally elevated SAMe levels and directly to liver disease (Avila et al., 2000; Luka et al., 2002; Mudd et al., 2001). All these data indicate that the liver needs to tightly regulate the amount of SAMe, since the impairment in its metabolism leads to liver injury. In the next sections, the main enzymes implicated in the regulation of SAMe, MATI/III (anabolism) and GNMT (catabolism), and the study of hypermethioninemia based in this two enzymes knock-out mouse models, will be described in detail.

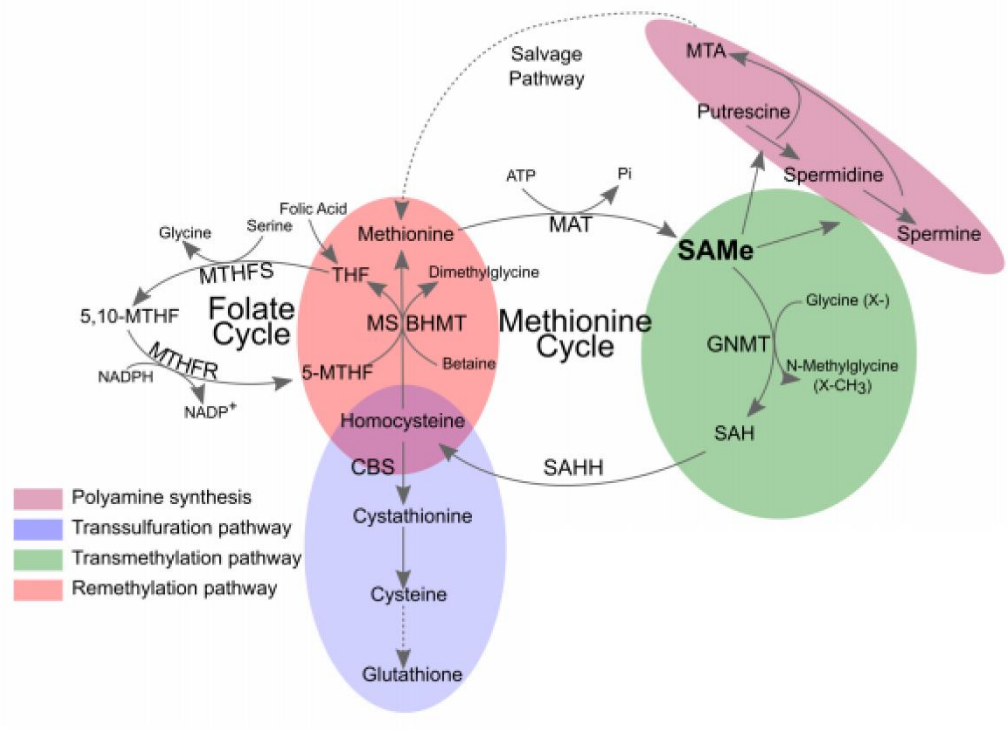

Figure 2.12 The methionine cycle. Brief overview of the methionine and SAMe metabolism in the liver and the pathways implicated in their use and regeneration.

2.2.2 METHIONINE ADENOSYLTRANSFERASE

MAT enzymes are the responsible of SAMe generation, activating methionine and using ATP. *MAT* gene is very well conserved from bacteria to humans and it is considered essential in order to sustain life (Kotb et al., 1997).

In mammals, MAT catalytic subunit is codified by two genes located in different chromosomes, *MAT1A and MAT2A,* encoding for the two homologous MAT catalytic subunits α1 and α2 (sharing 84% of amino acid homology), respectively (Kotb et al., 1997). A third MAT gene exists, *MAT2B*, only expressed in fetal and regenerating liver and extrahepatic tissues, which encodes for the β regulatory subunit (Halim et al., 1999). *MAT1A* is expressed in adult and differentiated liver (Gil et al., 1996). The encoded α1 subunit can be organized forming dimers (MATIII) or tetramers (MATI) (Kotb et al., 1997; Mato et al., 1997). *MAT2A* is expressed in fetal and proliferating liver and extrahepatic tissues (Gil et al., 1996). The encoded α2 subunit is organized as a tetramer (MAT II) that associates with the β regulatory subunit (LeGros et al., 2000; YANG et al., 2008). MAT enzymes are tightly regulated regarding expression and enzymatic activity.

a) Regulation of *MAT* expression

MAT1A and *MAT2A* are differentially expressed in several liver scenario. During fetal rat development, *MAT1A* expression increases very rapidly, being highly expressed in adult rat. Conversely, *MAT2A*, decreases during development and after birth, being minimally expressed in adult rat liver (Gil et al., 1996).

In HCC and different liver diseases, *MAT1A* is downregulated, while *MAT2A* is found overexpressed. In murine and rat liver, loss of *MAT1A* and increase *MAT2A* expression have been also found in situation of rapid growth, such as hepatocyte de-differentiation to fibroblast in culture (García-Trevijano et al., 2000) and liver regeneration after partial hepatectomy (Huang et al., 1998).

Different mechanisms implicated in the regulation of *MAT* genes in these situations have been described. The implication of DNA methylation has been described in different cases. *MAT1A* promoter is hypermethylated in two CpG sites in fetal liver and extrahepatic tissues, and have been found hypermethylated in cirrhosis, HCC and hepatoma cell lines (Avila et al., 2000; Mato et al., 2002). On the contrary, *MAT2A* promoter is hypomethylated in HCC (Yang et al., 2001). Other mechanisms such as histone acetylation (Latasa et al., 2001; Torres et al., 2000) and microRNA (Yang et al., 2013a) mediated repression have been described concerning *MAT* regulation in liver disease.

b) Regulation of MAT enzymatic activity

The three different MAT enzymes have different kinetic properties implicated in the regulation of its activity. MATII has the lowest K_m for he methionine (4-10 µM), MATI has intermediate (23 µM-1 mM) and MATIII possesses the highest K_m for the methionine (215 µM-7 mM) (Mato et al., 2013). The rate of inhibition by its product SAMe, shows an opposite trend: MATII is strongly inhibited by SAMe (IC_{50}= 60 µM); MATI (IC_{50}= 400 µM) is slightly inhibited and MATIII, on the contrary is strongly activated by SAMe (Sullivan and Hoffman, 1983). Other mechanisms implicated in the regulation of enzymatic activity are the production of nitric oxide (NO) and ROS, which can switch MATI and III to inactive conformational forms (Mato et al., 2002).

2.2.2.1 Mat1a-knock-out mouse model

Mat1a$^{-/-}$mice are characterized by the absence of *MAT1A* gene and the consequent lack of MATI/III enzymes (Lu et al., 2001). These mice have reduced SAMe levels (74% compared to WT mice) and GSH levels (40%) and increased blood levels of methionine

(Lu et al., 2001). The lack of *Mat1a* results in the spontaneous development of NAFLD and HCC at 8 and 18 months, respectively. *Mat1a*$^{-/-}$ mice present hyperglycemia and high levels of hepatic triglycerides (Lu et al., 2001) together with deficient VLDLs formation, which are smaller and with less TG content (Cano et al., 2011), contributing to the development of steatosis. Moreover, these mice show overexpression of CYP2E1 and UCP2, both related with ROS production, which together with the decreased GSH levels predispose these mice to the development of liver injury (Martínez-Chantar et al., 2002). Finally, these mice are characterized by increased expression of proliferating markers, such as PCNA, AFP and MATII, corresponding with increased proliferation. All these features predispose *Mat1a*$^{-/-}$ mice to the spontaneous development of HCC at 18 months.

2.2.3 GLYCINE N-METHYLTRANSFERASE

Glycine N-methyltransferase (GNMT) is the most important enzyme implicated in SAMe catabolism. GNMT is a tetrameric protein composed of four identical subunits that is mainly localized in the cytoplasm of the cell (Ogawa et al., 1998; Yeo and Wagner, 1994). In the liver, it represents about 1-3% of the total cytosolic protein content, and it is also expressed in the pancreas, prostate and peripheral nervous system (Luka et al., 2009; Varela-Rey et al., 2014). Similarly to *MAT1A*, *GNMT*, is not expressed during development and its expression rapidly increases after birth, representing an marker of adult and differentiated liver (Luka et al., 2009).

GNMT catalyzes the conversion of SAMe to SAH, using glycine as substrate and generating sarcosine, being considered to intracellular regulator of SAMe levels, maintaining the ratio SAMe/SAH (transmethylation capacity of the cell) constant. Under situations of high methionine levels in the cell, SAMe levels will be increased, and GNMT activation will metabolize SAMe. The resulting SAH is known to inhibit most methyltransferase, however, GNMT activity is not inhibited by high levels of SAH (Wagner et al., 1985), allowing a continued activity of GNMT in order to catabolize SAMe (Martinov et al., 2010; Rowling et al., 2002). On the other hand, the second product generated by GNMT is sarcosine, a molecule with unknown metabolic functions that can be used to regenerate glycine and 5,10-MTHF, linking GNMT with the folate cycle. In fact, GNMT was first described as a folate-binding protein, GNMT activity is inhibited by 5-MTHF under low methionine conditions, allowing the use of SAMe-methyl group to be used for the desired biological fate (Luka et al., 2009; Rowling et al., 2002; Wagner et al., 1985).

GNMT expression is frequently found downregulated in liver disease (Avila et al., 2000; Heady and Kerr, 1975; Liao et al., 2012; Luka et al., 2002; Mudd et al., 2001; Tseng et al., 2003). However, the mechanisms mediating GNMT downregulation are not profoundly described. Mechanism implicating GNMT promoter hypermethylation in hepatocellular carcinoma have been proposed (Huidobro et al., 2013), however, aberrant GNMT hypermethylation was only found in 20% of human HCC lacking GNMT expression, thus, explaining only partially GNMT downregulation. GNMT promoter acetylation has been proposed but not profoundly studied in HCC (Kant et al., 2016). Finally, GNMT activity has been proposed to be induced by Vitamin A, glucocorticoids and glucagon (Nieman et al., 2004; Williams and Schalinske, 2007). Thus, better characterization of GNMT expression in liver disease is necessary to overcome GNMT-deficient related liver injury.

2.2.3.1 *Gnmt-knock-out* mouse model

As previously mentioned, GNMT is frequently absent or low expressed in HCC and other liver diseases (NAFLD and cirrhosis), supporting an essential role of GNMT in liver homeostasis. Aiming to understand the functions of GNMT, the *Gnmt*$^{-/-}$ mouse has been generated (Luka et al., 2006). *Gnmt*$^{-/-}$ mice are characterized by 35-fold increased hepatic SAMe levels and 100-fold increase in SAMe/SAH ratio, as well as elevated serum levels of methionine an transaminases (Martínez-Chantar et al., 2008). *Gnmt*$^{-/-}$ mice develop steatosis and steatohepatitis and fibrosis at 3 months and hepatocellular carcinoma at 8 months (Martínez-Chantar et al., 2008) (Figure 2.13). This *Gnmt*$^{-/-}$ murine model has been extremely useful for the study of GNMT-deficiency associated diseases and SAMe excess in the liver. Different mechanisms contributing to liver injury in the absence of GNMT have been described (Figure 2.14):

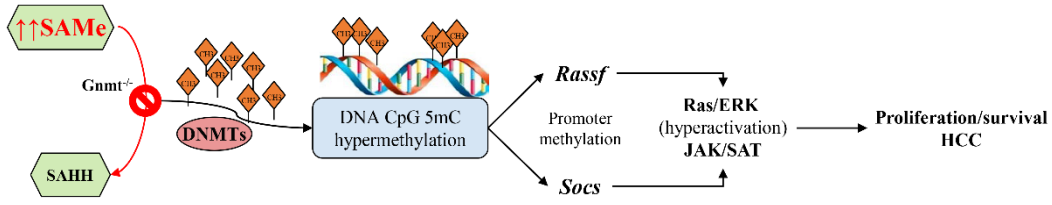

Figure 2.13 *Gnmt*$^{-/-}$ mice present DNA hypermethylation and develop HCC. GNMT deficiency leads to chronic accumulation of SAMe in the liver and hepatocellular carcinoma. SAMe excess is associated with a DNA hypermethylation signature. Gene promoter methylation of inhibitors of Ras/ERK and JAK/STAT pathways is produced in the absence of GNMT, and, as a consequence, these oncogenic pathways are overactivated in these mice contributing to HCC development.

a) DNA hypermethylation

The first characterization of *Gnmt*$^{-/-}$ mice identified a role of GNMT in the maintenance of normal DNA methylation. The absence of GNMT leads to chronic SAMe accumulation in the liver and the 100-fold SAMe/SAH. SAMe/SAH ratio is related to the methylation capacity of the cell, and its increase promotes aberrant methylation reactions. SAMe is able to methylate DNA at gene promoter. In these mice, the chronic excess of SAMe has been related to hypermethylation of Ras-association domain family (RASFF) and suppressor of cytokine signalling (SOCS) promoters. RASFF and SOCS are inhibitors of Ras and JAK/STAT signalling pathways. This inhibition results in Ras/MEK/ERK and JAK/STAT/CyclinD1/D2 signalling hyperactivation, increasing the proliferative and survival capacity of the cells, driving HCC development (Martínez-Chantar et al., 2008) (Figure 2.13).

b) Alterations in the PEMT flux

SAMe participates in the synthesis of around 30% of liver phosphatidylcholine (PC), in a reaction in which phosphatidylethanolamine (PE) is converted into PC through the action of the phosphatidylethanolamine N-methyltransferase (PEMT) enzyme. The flux from PE to PC is known as PEMT flux. PC is catabolised into diglycerides, a precursor for TG synthesis. Thus, the increase in SAMe in *Gnmt*$^{-/-}$ livers leads to increased PEMT flux and PC formation, contributing to increase TGs and steatosis in these mice (Martínez-Uña et al., 2013). Moreover, PEMT flux has been described to be essential for VLDL secretion from the liver (Noga et al., 2002). In this regard, the increased PEMT flux in *Gnmt*$^{-/-}$ mice due to excess of SAMe increases VLDL assembly and secretion but also VLDL uptake, mainly due to the VLDL specific features. Altogether, these alterations contribute to steatosis and some of the extrahepatic complications of NAFLD (Martinez-Una et al., 2015).

c) Alterations in autophagy

Excess of SAMe in *Gnmt*$^{-/-}$ mice has been related to the development of steatosis through disruption of lipophagy (Zubiete-Franco et al., 2016). Lipophagy is a type of autophagy implicated in the degradation of lipids. It has been proposed that autophagy can protect the liver from steatosis by eliminating the accumulation of lipids within the hepatocytes (Singh et al., 2009). High levels of methionine and SAMe inhibit autophagy through the methylation of PP2A, a protein implicated in inhibition of mTOR-mediated

autophagy. The inhibition of autophagy through this mechanisms leads to liver steatosis in *Gnmt*$^{-/-}$ mice (Sutter et al., 2013; Zubiete-Franco et al., 2016).

d) Hypercholesterolemia

Recently, the deficiency of GNMT has been shown to impact cholesterol metabolism, in this case, independently of SAMe metabolism. GNMT interacts with and stabilizes Niemann-Pick type C2 (NPC2), a protein implicated in cholesterol trafficking and metabolism through the binding with free cholesterol. Low GNMT hepatic levels decreases the stability of NPC2, contributing to cholesterol accumulation within the hepatocytes (Liao et al., 2012).

e) GNMT deficiency associated inflammation in steatohepatitis.

The role of GNMT and SAMe in the regulation of inflammatory processes and immune system have been described in two studies using *Gnmt*$^{-/-}$ mice. In a first study it was shown that GNMT deficiency and SAMe excess in the liver leads to overactivation of immune system, increasing the number and cytotoxic activity of TRAIL-producing NK/NKT cells in the liver, contributing to the chronic proinflammatory environment characteristic during NASH progression in the *Gnmt*$^{-/-}$ mice (Gomez-Santos et al., 2012). Finally, the role of TRAIL-producing NK cells in GNMT deficient mice was also described in the progression of chronic liver injury and fibrogenesis (Fernández-Álvarez et al., 2015).

f) Other signalling pathways contributing to HCC.

Despite the highlighted role of GNMT in the regulation of SAMe/SAH ratio and the methylation capacity of the cell, other mechanisms have been shown to be implicated in GNMT-deficient derived hepatocellular carcinoma, besides DNA promoter hypermethylation above described. In one study, *Gnmt*$^{-/-}$ mice showed hyperactivation of canonical Wnt pathways (Liao et al., 2009). Another study implicates GNMT as an interacting protein of DEPDC6/DEPTOR protein, regulating mTOR signalling and HCC (Yen et al., 2012). Finally, recently published data discovered PREX2 (an inhibitor of PTEN) as a novel interacting protein of GNMT. GNMT-PREX2 interaction enhances PREX2 proteasome degradation. Thus, lack of GNMT correlates with increased PREX2 levels, PTEN inhibition and hyperactivation of Akt signalling and HCC development (Li et al., 2017).

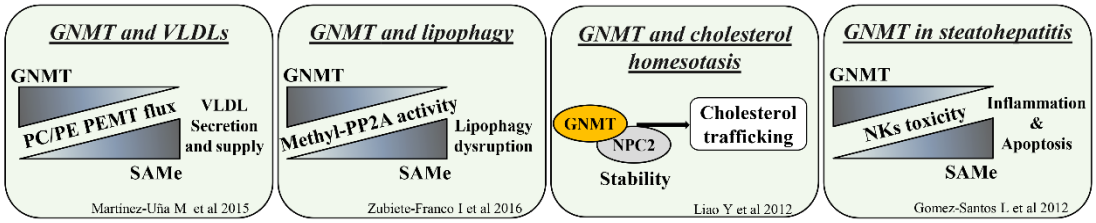

Figure 2.14 GNMT-deficiency associated alterations. Deficiency in GNMT have been associated with different liver dysfunctions such as alterations in VLDL secretion, lipophagy, cholesterol trafficking and deregulation of NK cell toxicity in the liver.

2.3 EPIGENETICS IN LIVER DISEASE

Alterations in SAMe metabolism caused by any possible disruption in the expression or function of the enzymes implicated in the methionine cycle are strongly associated to epigenetic modifications. Specifically, in the context of GNMT deficiency and excessive SAMe, epigenetic changes can involve DNA methylation and histone methylation. The term epigenetics involves a variety of regulatory processes implicated in the control of gene expression by operating "above" ("*epi*") the DNA sequence. The consensus definition of an epigenetic trait is "a stably inherited phenotype resulting from changes in a chromosome without alterations in the DNA sequence." Most important mechanisms of epigenetics include: DNA methylation, histone modifications, microRNAs and long noncoding RNAs, transcription factors and chromatin remodeling complexes. In the next sections, most important epigenetic mechanisms and their implications in liver disease will be described.

2.3.1 DNA METHYLATION

DNA methylation occurs in the cytosine, at the 5th carbon ring, mainly found within CpG islands, which are long stretches of DNA with dense sequences CpG (55%), frequently located in the 5' promoter regions. CpG methylation is associated with transcriptional repression, which is mediated by the union of methyl binding domain proteins (MBDs: MBD1, MBD2, MeCP2, KAISO and MBD4) that recognizes methyl-CpG regions in the DNA. Besides MBD union, methylation of CpG islands also inhibit DNA transcription promoting high chromatin condensation (Bird, 2002; Bird and Wolffe, 1999; Cedar and Bergman, 2012; Suzuki and Bird, 2008). Of note, DNA methylation out of the CpG islands is related to increased transcriptional activity, by mechanisms still not fully understood (Jones, 2012). DNA methylation is known to be established during embryonic development, becoming stable through the adult life, and it is maintained

through cell division (Smith and Meissner, 2013). Methylation of DNA is mediated by the enzymes called DNA methyltransferases (DNMTs), which transfer the methyl group of SAMe to DNA. DNMTs can be classified as *de novo* DNMTs (DNMT3a and 3b) or maintenance DNMT (DNMT1), regarding its DNA substrate. On one hand, DNMT1 is the responsible of the maintenance of the DNA methylation status during DNA replication and cell division. On the other hand, DNMT3a and DNMT3b methylate new CpG islands stablishing new epigenetic marks (Goyal et al., 2006; Jones and Liang, 2009; Klose and Bird, 2006; Okano et al., 1999). Recently, it has been described the role of cytosine oxidation in DNA methylation, 5-hyroxymethylcytosine. Hydroxymethyl CpG is an intermediate step in DNA demethylation and is associated to increased transcriptional activity. Oxidation of methyl-CpGs is produced by the activity of the Ten Eleven Translocation (TET) enzymes (TET1, TET2 y TET3) (Delatte et al., 2014; Tahiliani et al., 2009).

The role of DNA methylation in liver disease is one of the best characterized epigenetic mechanisms, especially in fibrosis and hepatocellular carcinoma (Figure 2.15-16).

a) DNA methylation in NAFLD

The study of DNA methylation in NAFLD is increasing in the recent years and it is mainly focused in the progression to fibrosis. One of the most important firsts studies in this field, identified differential DNA methylation in about 70000 CpG islands in liver biopsies from mild versus severe NAFLD, linking DNA methylation with the progression of NAFLD to fibrosis (Murphy et al., 2013). A similar study, using DNA methylation and transcriptomics analysis, has identified differentially methylated genes in healthy, obese and NASH patients (Ahrens et al., 2013). Some studies have identified aberrant methylation of important genes involved in NAFLD progression, such as PNPLA3 (Kitamoto et al., 2015), MT-ND6 (a mitochondrial NADH dehydrogenase) (Pirola et al., 2013), or PGC1α (Sookoian et al., 2010), and, more importantly, global mitochondrial DNA hypermethylation (Carabelli et al., 2011; Lee et al., 2017; Sun et al., 2015a), linking it with the progression and the severity of the disease. Moreover, in the context of NAFLD progression to fibrosis, genes involved in bile acid metabolism and detoxification (FXR, BSEP, CYPs, etc.) have been found aberrantly methylated, contributing to fibrosis development (Schiöth et al., 2016).

In another study, it was shown that DNA methylation status of key genes involved in fibrosis progression could stratify NASH patients according to the severity of the disease (Zeybel et al., 2015). Although these studies have been useful for identifying a role of DNA methylation in NAFLD progression, they present some major limitation: they reveal low mechanistic implications; presents impossibility of identifying DNA methylation as a cause of the disease and they were carried out in liver biopsies, which implicates that the changes observed in the whole liver can be due to cellular changes related to the fibrogenic process. In this regard, most recent study in the field has identified PPARγ promoter methylation in serum as an important predictor of fibrosis progression in NAFLD and a biomarker for disease severity (Hardy et al., 2017).

b) DNA methylation in fibrosis

Liver fibrosis (section 2.1.2) is driven by different mechanism and cell types among which, hepatic stellate cells (HSC) are the main contributors to extracellular matrix deposition and scar formation in the liver. During fibrosis, HSCs are activated through transdifferentiation acquiring a myofibroblast-like phenotype. This HSC activation may be orchestrated by different epigenetic mechanisms, such as transcription factors, DNA methylation, histone remodeling and miRNAs, allowing fast phenotypic changes in the cell. Changes in the DNA methylation landscape of HSCs during transdifferentiation have been study by different authors. Upon activation, HSCs express higher MeCP2 levels, associated to increased methylation of CpG islands (Mann et al., 2006, 2010; Meehan et al., 1992). MeCP2 is related to transcriptional repression. It has been show that MeCP2 together with the histone methyltransferase EZH2 (section 2.4.2.2) mediates the repression of PPARγ in HSCs, a master regulator of the maintenance of the quiescent phenotype in the HSCs (Mann et al., 2006, 2010). Moreover, MeCP2 binding to DNA in association to CBF1 mediates transcriptional repression of IκB (Mann et al., 2006; Oakley et al., 2005). Finally, MeCP2 has been also implicated in repression of PTCH1 in HSCs (Yang et al., 2013c) However, MeCP2 is not only implicated in transcriptional repression but also in activation. In this sense, MeCP2 induces the expression of ASH1, an histone methyltransferase expressed during HSCs transdifferentiation that induces profibrogenic gene expression (section 2.4.2.2) (Perugorria et al., 2012). Overall, these functions of MeCP2 together with the fact that MeCP2 deficient mice do not develop liver fibrosis, indicates MeCP2 is a master regulator of liver fibrosis.

A recently published study has further implicated DNA methylation in the control of liver fibrogenesis. In this study, it is demonstrated, in different experimental animal models of liver fibrosis, that DNA methyltransferases (DNMTs), DNMT1 and, specially, *de novo* DNMT3a and DNMT3b, are upregulated in the liver and HSCs during fibrogenesis initiation. On the contrary, TET enzymes are downregulated during this process. These observations are in accordance with the global changes in the DNA methylome produced during HSC activation, showing a tendency towards increased levels of 5-methylcytosine (5-mC) and a clear decrease of 5-hydroxymethylcytosine (5-hmC). Importantly, the results described in the experimental animal models were similar to epigenetic marks observed in fibrotic human patients in the same study (Page et al., 2016).

Despite HSCs are the main contributors to liver fibrosis and are the best characterized cell regarding its control by epigenetic mechanisms, not only HSC methylome is changed during fibrogenesis. Methylation have been described to affect the expression of important genes in hepatocytes, such as PPARγ (Hardy et al., 2017). The hypermethylation of different bile acid transporters and metabolism regulators above mentioned have been also described, affecting bile acid induced cholestasis and fibrosis (FXR, HNF4α, ABCG5, BSEP, etc.) (Schiöth et al., 2016).

c) DNA methylation in HCC

DNA methylation changes in HCC and other cancers has specially attracted the attention of researchers in the last years. Regarding cancer development, DNA methylation can play two opposite roles: hypomethylation of oncogenes leads to increased expression of oncogenic drivers, while hypermethylation of tumor suppressor genes contributes to cancer development through the downregulation of genes implicated in cancer progression blockage. Several studies of HCC have identified different signatures of DNA methylation in HCC at genome-wide levels, showing global alterations of variable number of hypo/hypermethylation of gene promoters (Nishida et al., 2012; Revill et al., 2013; Shen et al., 2012; Song et al., 2013; Stefanska et al., 2011; Villanueva et al., 2015). Some of these studies were able to stablish a correlation between some methylation signatures and prognosis and recurrence of HCC (Nishida et al., 2012; Villanueva et al., 2015). Nevertheless, the major limitation concerning genome-wide methylation studies is the fact that HCC is a very heterogenic cancer with a high number of cells implicated, and thus, detected changes in methylation could be only the

consequence of different cell population relative presence (Hardy and Mann, 2016). Moreover, some other studies more focused on the analysis of concrete gene promoter methylation have identified specific promoter hypermethylation of tumor suppressor genes frequently downregulated in HCC, such as CDKN2A, APC, DKK, SOCS, RASSF, p16 (INK4A), CASP8, ASC and GSTP1 among others, implicated in the inhibition of different pathways known to contribute to HCC development (Ras, JAK/STAT, Wnt, section 2.1.3.2.2), regulation of apoptosis and DNA repair (Calvisi et al., 2006, 2007; Kaneto et al., 2001; Kubo et al., 2004; Nishida et al., 2012; Niwa et al., 2005; Tischoff and Tannapfel, 2008; Yu et al., 2002). As previously mentioned, the promoters of MAT1A and GNMT have been described to be hypermethylated in HCC. Thus, both tumor suppressor genes highly implicated in the regulation of SAMe metabolism are susceptible of SAMe-mediated hypermethylation. Finally, as for the case of DNA methylation analysis mentioned in NAFLD and fibrosis, recent advances are providing increasing evidence suggesting the use of DNA methylation in serum as a marker of detection and prognosis in HCC (Hardy and Mann, 2016; Shen et al., 2012; Snyder et al., 2016; Xu et al., 2017)

According to increased promoter methylation, DNMT (DNMT1, DNMT3a and DNMT3b) overexpression in HCC have been described by different authors. However, a clear correlation between promoter methylation and the DNMT expression levels has not been stablished (Lim et al., 2008; Nagai et al., 2003; Park et al., 2006; Saito et al., 2001, 2003; Tischoff and Tannapfel, 2008). Conversely, some studies have described TET enzymes downregulation in HCC, contributing to the mentioned changes in DNA methylation (Chen et al., 2017; Chuang et al., 2015; Liu et al., 2013; Yang et al., 2013b).

2.3.2 HISTONE MODIFICATIONS

DNA must be packed in the nucleus to form chromatin in two different states, heterocromatin (highly packed) and euchromatin (lightly packed). This package is mediated by the histone proteins and must be differentially regulated depending on the transcriptional necessity of the cell. The most basic structure of chromatin is the nucleosome, consisting on 147 base pair of double stranded DNA wrapped around a core of eight histones (H2A, H2B, H3 and H4, each of them twice). The core of histones has an N-terminal amino acid tail that can be the target of different covalent post-translational modification (methylation, acetylation, phosphorylation, ubiquitination, sumoylation and ADP-rybosilation), which in turn will control the grade of package of the chromatin,

promoting or suppressing the transcription (Clapier and Cairns, 2009). Moreover, histone modifications are highly related to chromatin spacing and packaging remodeling, processes carried out by different protein complexes (SWI/SNF and the polycomb group, PcG, respectively) that determine the grade of accessibly of transcription factors to the nucleosome, i.e. the transcription of DNA (Clapier and Cairns, 2009; Margueron and Reinberg, 2011; Mohrmann and Verrijzer, 2005).

Histone methylation and acetylation are one of the best characterized modification in liver disease. Histone acetylation promotes a chromatin relaxed state, correlating with increased transcriptional activity, and it is controlled by the balance between histone acetyltransferases (HAT) and histone deacteylases (HDAC). Dysregulation of HDACs and histone acetylation have been proved in several studies in NAFLD, fibrosis and HCC. Moreover, the benefit of HDAC inhibitors has been demonstrated in different models of liver disease (Armeanu et al., 2005; Barbier-Torres et al., 2015; Elsharkawy et al., 2010; Kirpich et al., 2013; Lee et al., 2017; Mannaerts et al., 2010; Niki et al., 1999; Pathil et al., 2006; Van Beneden et al., 2013).

As mentioned before, proteins are one of the known substrates of SAMe for methylation (Section 2.2.1). Histones are among the group of proteins that can be methylated by SAMe (Ara et al., 2008; Shyh-Chang et al., 2013), thus, influencing chromatin accessibility and DNA transcription. Histones can be methylated in different lysines, exerting opposite effects depending on the lysine residue methylated. H3 trimethylation in lysine 4 (H3K4me3) and lysine 6 (H3K6me2/3) are associated with light packed chromatin and gene expression, while H3 trimethylation in lysine 9 (H3K9me3) and 27 (H3K27me3) are associated with gene silencing (Black et al., 2012; Martin and Zhang, 2005). Histone methylation at different residues is mediated by histone methyltransferases, whose contribution to liver disease have been specially characterized in liver fibrosis and HSCs transdifferentiation. During HSCs activation, DNA methylation changes are frequently accompanied by changes in histone methylation and histone methyltransferases expression. As previously mentioned, the histone methyltransferase EZH2 is known to play an important role in the repression of PPARγ together with MeCP2, mediating H3K27me3, which is crucial for HSC activation (Mann et al., 2010). Another important histone methyltransferase in liver fibrosis is ASH1 (methylates H3 at lysine 4 and 36), which accumulates at the promoter region of *COL1A1*,

αSMA, *TIMP1* and *TGFβ1* genes, enhancing its transcription and the consequent development of liver fibrosis (Perugorria et al., 2012).

2.3.3 MicroRNAs IN LIVER DISEASE

The last epigenetic modification that can control gene expression is governed by microRNAs (miRNA, miR). MicroRNAs are small non-coding RNAs (21-25 nucleotides) that regulate gene expression at the posttranscriptional level by mRNA repression or degradation. Gene regulation by microRNAs takes places through complementarity base pairing with the mRNA, being that one microRNA can target thousands of mRNAs, as well as one mRNA can be targeted by thousands of microRNAs. In the recent years, microRNAs have been implicated in the control of many biological process such as proliferation, cell cycle control, metabolism, apoptosis and tumorigenesis. Importantly, alterations in microRNA levels have been described in many human diseases and cancer (as for the case of liver disease). The discovery and characterization of microRNAs have improved the understanding of the pathogenesis of diseases, emerging as useful targets for intervention and also as powerful disease-associated markers in tissue and serum. In the liver, dysregulation of microRNAs has been related with NAFLD development, fibrosis and liver cancer.

The next sections will summarize the basic aspects of microRNA-mediated gene repression and the role of microRNAs in liver disease.

2.3.3.1 MicroRNA regulation and gene repression

MicroRNA synthesis is a well characterized process in which 6 sequential steps take place to produce the mature microRNA. MiRNAs are transcribed from the host gene to large RNA precursors (pri-miRNAs) by RNA polymerase II. Pri-miRNAs are then processed by the canonical microprocessor complex (formed by the RNase III, Drosha and the double-stranded-RNA-binding protein, Phasa/DGCR8, DroshaDGCR8), resulting in a ~70 nucleotide hairpin-like sequence called pre-miRNA. Pre-miRNAs are exported from the nucleus to the cytoplasm by the exportin 5 and Ran-GTP complex, where they are additionally processed by the RNase III, DICER, generating a double-stranded RNA (~22 nucleotides) composed by the mature miRNA and complementary sequence. DICER also induces the formation of the RNA-induced silencing complex, the RISC complex, where only the mature miRNA (single-stranded) coming from the double-stranded miRNA is finally loaded and directed to the target mRNA. Once in the RISC

complex, mature single-stranded miRNA targets the mRNA at complementary sequences within the 3' untranslated regions (UTRs), interacting with Argonaute members, forming the miRISC, where the target mRNA is repressed through different mechanism, such as deadenylation, degradation or translational repression. The selection of the mature miRNA strand is controlled by the Argonaute proteins and it is mainly based on the stability of the sequence. The complementary strand of the miRNA is degraded by the RISC complex (Ambros, 2001; Filipowicz et al., 2005; Ha and Kim, 2014; Jonas and Izaurralde, 2015; Shukla et al., 2011; Wang et al., 2012) (Figure 2.16).

MicroRNAs and the above described regulatory mechanism of microRNA synthesis and mRNA targeting it is known to be a highly evolutionary conserved mechanism (Krol et al., 2010). The importance of microRNAs in the regulation of many biological process and its link to diseases and cancer development indicates microRNAs levels must be tightly regulated by strictly controlled processes. The regulation of microRNAs can be controlled by transcriptional activation or repression (Calin et al., 2004; Dews et al., 2006; Marson et al., 2008; O'Donnell et al., 2005; Woods et al., 2007) and by its own promoter hypermethylation (Lujambio et al., 2008; Shen et al., 2012; Toffanin et al., 2011).

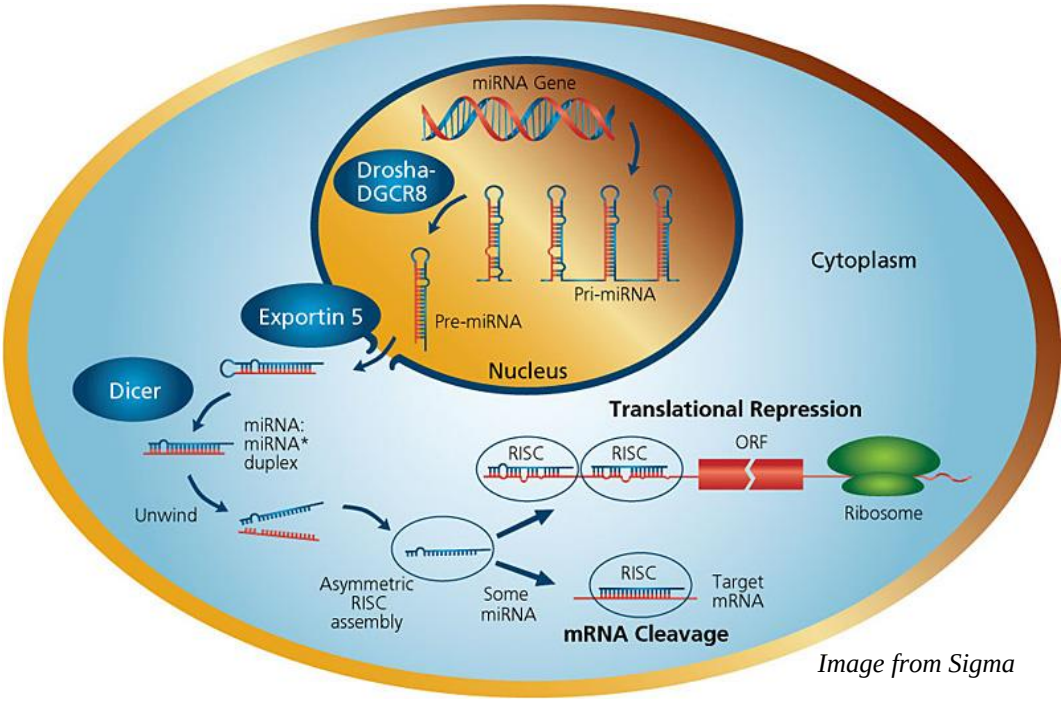

Image from Sigma

Figure 2.16 Overview of microRNA biogenesis and mRNA targeting.

2.3.3.2 MicroRNAs in liver disease

Since the publication of the first study describing a link between a microRNA and cancer (Calin et al., 2002), the number of studies aiming to identify new microRNAs and target genes implicated in the regulation of different diseases, and specially cancer, has increased exponentially in the last decade. As for other disease, microRNAs have been largely studied in liver cancer progression as well as NAFLD and fibrosis. Several microRNAs have been described to orchestrate liver development, directing the fate of embryonic progenitor cells through the regulation of gene expression. For example, miR-122, the most abundant (70%) miRNA in liver is not expressed in embryonic liver, while its expression is switched on during liver development, regulating the expression of different transcription factors implicated in proliferation and cell differentiation (Chang et al., 2004; Deng et al., 2014; Lagos-Quintana et al., 2002).

In the case of NAFLD, microRNAs are emerging as important regulators of disease progression, regulating different pathways contributing to this disease, as lipid metabolism, lipogenesis, insulin resistance, lipoapoptosis and inflammation (Finch et al., 2014). Several microRNAs have been identified up-downregulated in human NAFLD patients and in animal NAFLD models. Moreover, microRNA detection in serum of NAFLD patients is starting to be extremely useful as non-invasive biomarkers of the disease (Ceccarelli et al., 2013).

MicroRNAs are also involved in the development of liver fibrosis and cirrhosis. Changes in the expression of different microRNAs and target genes have been identified in liver and in the different cells implicated in this disease. For example, downregulation of miR-29 family members results in HSCs activation and liver fibrosis (Roderburg et al., 2011). In parallel, miR-122 downregulation contributes to cytokine-mediated activation of HSCs (Hsu et al., 2012). Thus, the regulation of microRNAs in different cells is highly involved in the development of liver fibrosis. The contribution of microRNAs has been more profoundly studied in HSCs, as they are the major cells contributing to fibrosis. In HSCs, up-downregulated microRNAs have been proposed for different roles, including their activation, transdifferentiation, proliferation, migration and apoptosis (Kitano and Bloomston, 2016).

The implication of microRNAs in liver cancer and in concrete in hepatocellular carcinoma, has been the most studied field in the recent years. Different studies have identified important numbers of microRNAs downregulated (tumor suppressor miRNAs)

and upregulated (oncomiRs) in HCC. Important microRNAs frequently downregulated in HCC are the miR-122 (highly implicated in all manifestations of liver disease), miR-26, let-7 and miR-199. On the other hand, frequently microRNAs overexpressed in HCC (oncomiRs) are miR-151, miR-221, miR-21, miR-17-92 family and C19MC microRNA family members (miR-516-520), which are specifically characteristic of a subclass of HCC (Finch et al., 2014; Pineau et al., 2010; Toffanin et al., 2011; Wang et al., 2012). Moreover, the levels of some of these microRNAs are related to tumor recurrence, malignancy, invasion and metastasis, clinical outcome of patients or resistance to apoptosis and HCC treatment. Concerning the latter, microRNA profiling is being studied in the recent years aiming to identify microRNAs involved in drug resistance and in particular, sorafenib drug resistance. Some examples highlighting the potential of miRNA management in overcoming drug resistance involve the miR-122, miR-221 and miR-21 (Bai et al., 2009; Fornari et al., 2009, 2017; Xia et al., 2013; Xu et al., 2016), but the number of studies identifying new microRNAs and target genes involved in drug resistance in HCC is increasing in the last years.

Finally, the use of microRNAs as non-invasive biomarkers of liver disease is an important field of research. MicroRNAs are highly stable in cells and in circulation and can be isolated from serum, plasma and other body fluids. Thus, the detection of microRNAs in circulation is of special interest diagnosis. Despite the expectative concerning microRNAs as diagnostic tools for liver disease, there are some concerns limiting their use, as the relative difficulty of isolation and purification and the discrepancy regarding quantification methods.

2.3.3.3 Targeting microRNAs in liver disease

The broad regulatory roles of microRNAs in different liver processes and disease has positioned them in the spotlight of targeting therapy development. There are two different ways of targeting microRNAs, inhibiting or mimicking them, each one with their respective limitations and advantages. MicroRNA-mimics are based on short RNA duplex that mimics a microRNA, restoring to normal values the levels of the target mRNA. However, mimic-microRNAs present the major limitation of potentially targeting other mRNAs besides the desired target. On the other hand, microRNA-inhibitors are chemically modified single-stranded oligonucleotides that antagonize a microRNA by base complementarity, sequestering or degrading the microRNA. Major advantages of microRNA inhibitors are the low concentration required for their effect and

low toxicity, while major limitations concern the possibility of off-target effects over microRNAs belonging to the same family (Wang et al., 2012).

Despite the mentioned inconveniences of targeting microRNAs for therapeutic intervention, the interest in microRNA research have increased during the last years. Some studies, especially in complex diseases, as they are NAFLD, fibrosis and liver cancer, have demonstrated that targeting one microRNA can be highly beneficial in order to regulate at the same time the expression of different genes, sometimes associated to a complex regulatory network. In these situations, the possibility of targeting different mRNAs with one microRNA becomes an advantage instead of a problem, resulting easier than the conventional silencing or overexpression of a single gene.

3 HYPOTHESIS AND OBJECTIVES

3 HYPOTHESIS AND OBJECTIVES

The work presented in this thesis deals with the study of the regulation of GNMT by different microRNAs in the liver and its role in liver pathology in different manifestations of hepatic disease. In concrete, we have studied the impact of miR-873-5p and miR-518d-5p in Non-Alcoholic Fatty Liver Disease, in cholestasis induced fibrosis and in hepatocellular carcinoma.

Over the last years, several studies have highlighted the importance of methionine and SAMe metabolism in the liver. Dysregulation of SAMe metabolism and enzymes implicated in the methionine cycle have been reported in different liver diseases, as for the case of GNMT, the most important enzyme responsible for SAMe catabolism. GNMT is frequently found downregulated in liver disease, including NAFLD, cholestasis, fibrosis, cirrhosis and HCC, being considered a tumor suppressor in the liver and an interesting target for the study and design of liver disease therapies.

Despite the importance of GNTM in maintaining liver function and its frequent downregulation in liver disease, few mechanisms implicated in GNMT downregulation have been described. Moreover, these described mechanisms can only be considered in some specific liver diseases or have not even been described to occur in human pathology. Thus, we believe that the identification of general mechanisms mediating GNMT downregulation in different disease scenarios would be of high interest for the development of new liver disease targeted therapies.

During the last decade, microRNAs have emerged as important epigenetic regulators that mediate posttranscriptional gene repression and whose expression is altered in different diseases, such as cancer and in the liver in NAFLD, cirrhosis and HCC. The targeting of microRNAs in several preclinical studies has been shown as a promising strategy for the development of therapies. The main advantage of microRNA targeting, particularly in liver disease, relies in the fact that targeting a single microRNA can result in the regulation of different target genes, sometimes controlling or implicated in the control of the same pathway.

On this basis, we hypothesize that diverse microRNAs can be targeting GNMT in different liver diseases and that the regulation of these microRNAs could represent a

potential interesting therapeutic approach. Thus, the principal aims of this thesis are as follows,

Aim 1. To identify microRNAs targeting GNMT in human and murine liver, studying the expression levels of these microRNAs in association with liver diseases where GNMT is downregulated.

Aim 2. To study microRNAs implication in NAFLD through the repression of GNMT and its role in liver lipid metabolism.

Aim 3. To identify and characterize the implication of microRNAs targeting GNMT in cholestasis induced fibrosis.

Aim 4. To investigate the role of microRNAs and GNMT in HCC progression and study their link with HCC response to sorafenib treatment.

4 EXPERIMENTAL PROCEDURES

5 RESULTS

6 DISCUSSION

7 CONCLUSIONS

7 CONCLUSIONS

1. GNMT expression is essential for liver health and its downregulation is frequently found in different chronic liver disease manifestations, including NAFLD, cholestasis, fibrosis, cirrhosis and hepatocellular carcinoma, acting as an important driver of the disease.
2. GNMT is regulated by the microRNAs miR-873-5p and miR-518d-5p in NAFLD and fibrosis and in liver cancer, respectively.
    a) MiR-873-5p inhibition recovers GNMT levels in the mitochondria, regulating Complex II activity in the ETC potentially through sarcosine metabolism. This regulation results in decreased mitochondrial dysfunction and enhanced fatty acid β-oxidation protecting from NAFLD progression.
    b) In cholestasis, there is a broadly effect of miR-873-5p in the different cell populations implicated in hepatocytes apoptosis, ductular reactions, inflammation and HSC activation mediated by epigenetic mechanism.
    c) MiR-518d-5p levels in liver and serum correlate with the prognosis of HCC and mediates GNMT downregulation.
    d) MiR-518d-5 levels can predict HCC response to sorafenib and its inhibition overcomes sorafenib resistance.
    e) MiR-518d-5p targeting sorafenib induced apoptosis is mediated by increased ROS and mitochondrial dysfunction through the direct regulation of GNMT as well as c-Jun and its downstream target PUMA.
3. Targeting microRNAs regulates GNMT expression alongside other genes and pathways implicated in liver injury, underscoring the benefit of microRNA-based therapy versus conventional gene-specific-targeted therapies.

8 BIBLIOGRAPHY

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