Hepatocellular carcinoma in the context of non-alcoholic steatohepatitis (NASH):

Recent advances in the pathogenic mechanisms

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Abstract

Hepatocellular carcinoma (HCC) is the most common type of liver cancer. HCC is particularly aggressive and is one of the leading causes of cancer mortality. In recent decades, the epidemiological landscape of HCC has undergone significant changes. While chronic viral hepatitis and excessive alcohol consumption have long been identified as the main risk factors for HCC, non-alcoholic steatohepatitis (NASH), paralleling the worldwide epidemic of obesity and type 2 diabetes, has become a growing cause of HCC in the United States and Europe. Here we review the recent advances in epidemiological, genetic, epigenetic and pathogenic mechanisms as well as experimental mouse models that have improved the understanding of NASH progression towards HCC. We also discuss the clinical management of patients with NASH-related HCC and possible therapeutic approaches.

Abbreviations

ACC, acetyl-CoA carboxylase 1	NAFLD, non-alcoholic fatty liver disease
AR, androgen receptor	NASH, non-alcoholic steatohepatitis
BCLC, Barcelona Liver Clinic Classification	NKT, natural killer T
BI-1, bax-inhibitor 1	NLRP3, NOD-like receptor family, pyrin domain containing 3
CDD, choline-deficient diet	PAMP, pathogen-associated molecular pattern
CHOP, C/EBP homologous protein	ROS, reactive oxygen species
CCRK, cycle-related kinase	SASP, senescence-associated secretory profile
DAMP, damage-associated molecular pattern	SREBP, sterol regulatory element-binding protein
DCA, deoxycholic acid	STZ, streptozotocin
DEN, diethylnitrosamine	TACE, transarterial chemo-embolization
DNL, <i>de novo</i> lipogenesis	TLR, Toll-like receptor
ER, endoplasmic reticulum	TG, triglycerides
FFAs, free fatty acids	T2DM, type 2 diabetes
FXR, farnesoid X receptor	UPR, unfolded protein response
GF, germ-free	VEGF, vascular endothelial growth factor
GPIb α , glycoprotein 1b alpha	WD, western diet
HBV, hepatitis B virus	
HCC, hepatocellular carcinoma	
HCV, hepatitis C virus	
HFD, high-fat diet	
HSC, hepatic stellate cells	
IL, interleukin	
ICI, immune check point inhibitors	
IR, insulin receptor	
IRE1, inositol-requiring enzyme 1	
IRS, insulin receptor substrate	
LPS, lipopolysaccharide	
MCDD, methionine-choline-deficient diet	
miRNA, microRNA	

Introduction

Hepatocellular carcinoma (HCC) is the most frequent malignant liver tumour worldwide, ranking at the second and fifth positions in terms of incidence (> 840 000 cases) and mortality (> 781 000 deaths), respectively (data from Globocan 2018; <https://gco.iarc.fr>). This highly fatal cancer originates from mature hepatocytes or stem cell/progenitor hepatic cells. Until now, resection and liver transplantation are the main curative treatments but a small percentage of patients benefit. Due to frequent tumour relapse, survival after resection only reaches 40 to 70% (1). For patients with advanced HCC, two multi-target therapies, sorafenib and lenvatinib, have been approved by Food and Drug Administration as first-line treatments but they have modest effects on overall survival (2).

During the past three decades, the incidence of HCC has increased by 3-fold in North America and several European countries (3). While chronic infections with hepatitis C virus (HCV) and excessive alcohol consumption contributed substantially to HCC cases in these countries, it appears that a significant proportion of recently diagnosed cases of HCC are unrelated to these etiologies. Non-alcoholic fatty liver disease (NAFLD) which is frequently associated with obesity and type 2 diabetes mellitus (T2DM) has been identified as a new cause of chronic liver disease and as a risk factor for HCC in Western countries. NAFLD prevalence is estimated at around 24% in the general population as reported in a recent meta-analysis of population studies conducted essentially in Western countries (4). Due to the recent advancements in the field of anti-HCV therapies (5), it is likely that NAFLD will become the major cause of chronic liver disease and HCC in the next 30 years in these countries (6).

NAFLD encompasses the entire spectrum of fatty liver diseases occurring in the absence of significant alcohol consumption and ranging from nonalcoholic fatty liver (NAFL) to nonalcoholic steatohepatitis (NASH). NAFL is characterized by fat accumulation in hepatocytes. While simple steatosis can remain as a benign and asymptomatic disease which may regress after dietary changes and physical activity (7), the disease can progress slowly to NASH with the appearance of lobular inflammation, hepatocyte injury (ballooning) and various degrees of fibrosis. It is considered that about 30% of patients with NAFL progress to NASH (8). In the US population of patients with NAFLD, up to a third have NASH (20 million). A recent meta-analysis reports that the global prevalence of NAFLD and NASH among patients with T2DM are 55.5% and 37.3%, respectively (9). NASH is a major risk factor for liver-related morbidity and mortality due to its potential progression to cirrhosis, hepatic decompensation and HCC. The cumulative incidence of HCC mortality in patients with NASH-related

cirrhosis ranges between 0.4% and 12.8% per year (10-12).

The description of the pathogenesis of NAFL and NASH is outside the scope of this review and has been reported elsewhere recently (13). Briefly, obesity and T2DM are characterized by perturbations in the homeostasis of multiple organs including adipose tissue, liver, and intestine. This contributes to the establishment of adipose tissue-liver and gut-liver axes that play a prominent role in the pathophysiology of NAFLD. Thus, in obesity and T2DM, adipose lipolysis is enhanced which promotes the release of free fatty acids (FFAs) and glycerol into the circulation. Visceral adiposity in obesity is accompanied by inflammation and secretion of pro-inflammatory adipokines which convey to the liver. The ectopic uptake and deposition of lipids in the liver promotes the accumulation of triglycerides (TG) and gluconeogenesis. It is now widely admitted that lipid accumulation by itself is not sufficient to induce NASH but fatty livers are more susceptible to injury. The multiple-hit theory suggests that environmental factors (diet, lifestyle, gut microbiota) act in concert with genetic and epigenetic backgrounds to promote hepatic insulin resistance, chronic hepatitis and necroinflammation. Metabolic imbalance together with endoplasmic reticulum (ER) and oxidative stress, lipotoxicity (induced by non-esterified cholesterol, ceramides or diacylglycerol), and the presence of pathogen-associated molecular patterns (PAMPs) (facilitated by increased intestinal dysbiosis and gut permeability) lead to hepatocyte damage and death, immune cell infiltration, fibrogenesis and disease progression. The mechanisms leading to cancer development in this context are very complex and not entirely understood. However, one might consider that combination of aberrant metabolism and inflammatory environment sensitize to hepatocyte proliferation, DNA damage and arising gene mutations, that collectively foster neoplastic transformation.

The premise for this review is to provide recent status of knowledge on cellular and molecular events occurring at the transition from NASH/HCC. We emphasize epidemiological, genetic, epigenetic and pathophysiological evidences as well as experimental mouse models that led to a better understanding of NASH progression towards HCC (Figure 1). The clinical management of patients with NASH-related HCC as well as the possible therapeutic approaches are also discussed.

Epidemiology of NASH-related HCC

Obesity and T2DM confer a twofold increased risk of HCC-related mortality (14). Although the risk of HCC due to NAFLD is lower than that due to chronic HCV infection (yearly cumulative incidence of 2.6%

and 4%, respectively) (11), the growing epidemics of obesity and DM worldwide raises cause for concern. NASH-related HCC is expected to become increasingly prevalent (6). In 2010, NASH has been identified as the most common underlying etiologic risk factor for HCC in the US (59% of cases) (15). In addition, NASH-related HCC was the second leading etiology among patients awaiting liver transplantation in 2015 (21% of cases) (16) and is predicted to become the leading indication in the next coming years (17, 18). The increase in incidence of NASH-related HCC has been also well documented these past years for European countries such as the UK (19) and France (20).

Due to the burden of NAFLD-related liver diseases, there is an urgent need to better inventory these diseases to predict their medical, societal and economic impacts across countries. To address these important questions, mathematical dynamic Markov models of NAFLD and NASH have been recently developed based on data for adult prevalence of obesity and T2DM in several countries (21-23). For example, it has been estimated that there are 64 million individuals with NAFLD in the US and 52 million in the European countries (France, Germany, Italy, United Kingdom) (21). In all these countries, the prevalence of NASH among individuals with NAFLD should increase in the coming decades notably due to an aging population. As a consequence, the prevalence of NAFLD-related HCC is estimated to increase between 2016 and 2030 all over the world (47% for Japan; 86% for China; 93% for the UK; 108% for Germany; 125% for France; 130% for the US). By 2030, China is projected to have the higher incidence of NAFLD-related HCC cases annually (82% increase with 12,780 cases). Japan should experience the smallest increase (44% increase with 1,520 cases). In the US, the incidence of HCC is predicted to increase by 122% (12,240 cases). In Europe, France is projected to have the largest increase of HCC incidence (117%) (1,200 cases), while the UK should record the smallest one (88%; 1,600 cases) (22, 23).

Genetics and epigenetics in NASH-related HCC

Genetic predisposition. Inherited factors and ethnicity influence NAFLD susceptibility and progression. A higher rate of NASH is observed among Hispanic individuals with NAFLD while a lower rate is observed in non-Hispanic black individuals (24, 25). Ethnic variations have been related to polymorphism in *PNPLA3* (encoding the patatin-like phospholipase domain-containing protein 3, also known as adiponutrin) among others (26, 27). In addition, the presence of the rs738409 C>G polymorphism in *PNPLA3* is associated with a greater risk of progression of NASH to HCC (28, 29).

Driver mutations. With the development of large-scale techniques such as whole-exome sequencing analysis, the mutational spectrum of a large number of human HCC with different etiologies has been characterized. Several driver mutations have been identified including *TERT*, *TP53*, *CTNNB1* and *ARID1A* mutations (30, 31). However, no somatic mutation specific to NASH-related HCC has been identified yet. Additional studies are required to draw firm conclusions.

Gene methylation. A recent genome-wide DNA methylation analysis has identified alterations in DNA methylation that are specific to NASH liver tissue and NASH-related HCC and not detected in samples associated with HBV or HCV infection (32). This suggests that NASH-specific DNA methylation patterns may participate in NASH-related multistage hepatocarcinogenesis. In addition, a reduction of global and gene-specific histone H4K16 acetylation has been reported in NASH-related hepatocarcinogenesis in mice that induces silencing of genes related to cell death (33).

Non-coding RNA. Several non-coding microRNA (miRNA) have been reported to be dysregulated during NASH and the transition to HCC (34). miR-122 is a conserved liver-specific miRNA, representing about 70% of total hepatic miRNA in the adult mouse and playing an important role in cholesterol metabolism and hepatocyte differentiation. Using miRNA microarray analysis, it has been reported that miR-122 is among the most downregulated miRNA in the livers from patients with NASH (35). The knockdown of miR-122 in mice is associated with spontaneous development of NASH and progression to HCC in about 32% of mice after 12-17 months (36, 37) indicating that miR-122 is a critical contributor to the maintenance of liver homeostasis through metabolic, anti-inflammatory and anti-tumorigenic functions. At the opposite, miR-34a is among the most frequently elevated miRNA in the livers from patients with NASH and mice fed with a high-fat diet (HFD) (35). miR-34a targets several key metabolic actors among which the transcription factor hepatocyte nuclear factor 4 alpha (HNF-4 α) leading to increased hepatic TG levels (38). Interestingly, miR-34a expression is induced in the course of β -catenin-induced hepatocyte transformation and its specific inhibition with a locked nucleic acid-based inhibitor inhibits tumour development (39).

Using next-generation RNA sequencing, it has been shown that three other miRNA are differentially regulated (miR-301a-3p and miR-34a-5p increased and miR-375 decreased) with NAFLD progression in the livers of obese patients (40). Since similar patterns of miRNA expression are observed in corresponding HCC, this reinforces the hypothesis that miRNA dysregulations may favour NAFLD progression to HCC.

It has been demonstrated that the adipose tissue constitutes a major source of miRNA containing exosomes that have systemic effects and may regulate gene expression in distant organs such as the liver (41). It is therefore conceivable that in the event of fat mass alteration as observed in obesity, adipose-derived exosomes are modified in terms of quantity and quality and participate in the establishment and progression of NASH. While the role of adipocyte-derived exosomes in the development of HCC has not been clarified in the context of NASH, it has been recently reported that adipose tissue from obese mice secrete higher levels of exosomal noncoding circular RNA, which favour HCC growth in an experiment model (42).

Preclinical models of NASH-driven HCC

Due to limitations in access to human NAFLD liver tissue, the need for mechanistic understanding of NASH-driven HCC has promoted the development of preclinical mouse models. The ideal mouse model should present the pathological spectrum from steatosis to steatohepatitis with fibrosis and the subsequent development of HCC in a context of insulin resistance, hyperinsulinemia and overweight. The histopathological criteria that should be taken into account when working with a mouse model of NASH-driven HCC have been comprehensively reviewed elsewhere (43). Only a few available murine models recapitulate most of the human features. In addition, gene expression profiles between liver tissues from nine mouse models and patients with different stages of NAFLD have been compared and very little overlap has been found (44).

Dietary models. Several dietary models have been developed in mice to model NAFLD. However, few of them closely recapitulate the full spectrum of metabolic and histologic features of the human disease and lead to HCC. It is important to mention that mouse susceptibility to diets with regards to NAFLD and HCC development is highly dependent upon the mouse strain and its specific genetic background. HFD in which 60% of the caloric value is provided by saturated fats causes obesity and insulin resistance but simple hepatic steatosis in C57BL/6J mice (45). A higher degree of hepatic injury is obtained with western diet (WD) mimicking fast food menus (high saturated fat, fructose and cholesterol). However, the WD-based models do not progress to HCC (46). A diet high in *trans*-FA and fructose coupled with sedentary lifestyle has been reported to promote NASH and HCC with a high penetrance (60% of mice after 12 months) (47). The choline-deficient (CDD) and methionine/choline-deficient (MCDD) diets which favour hepatic lipid sequestration induce histological features of NASH but treated mice do not develop

insulin resistance, massively lose body weight and even develop cachexia (48). Recently, it has been reported that the combination of CDD with HFD avoids weight loss and induces liver disease progression to HCC in about 25% of mice after 12 months (49).

Models combining diets and toxins. The combination of toxins to diets has been reported to boost the development of NASH and HCC in mice. Thus, the combination of diethylnitrosaline (DEN), a chemical carcinogen promoting HCC development in mice, to HFD (50) or CD-HFD (51), increases HCC development notably by enhancing the production of pro-inflammatory cytokines. More recently, the addition of carbon tetrachloride, a widely used inducer of liver injury and fibrosis, to WD recapitulates the progressive metabolic and histologic features of NAFLD within 12 weeks and leads to HCC development by 24 weeks in 100% of mice (52). The STAM mouse model combines the administration of streptozotocin (STZ) after birth followed by HFD at 4 weeks of age (53). In this model, NASH is detected after 7 weeks, followed by fibrosis at 9 weeks and HCC after 16 weeks with a high penetrance (100%). However, HCC develops in a context of insulinopenia due to STZ-induced pancreatic islet β -cell injury and in the absence of weight gain.

Genetics models. *Ob/ob* (leptin deficiency) and *db/db* (leptin receptor mutation) mice are two widely used models of genetically-induced obesity associated with steatosis and insulin resistance. Unless being treated with DEN (50), these animals develop neither NASH, nor spontaneous HCC. Recently, DIAMOND mice (hybrids obtained from isogenic cross between B6/129 strains) have been selected for their strong susceptibility to NASH and HCC (HCC incidence of 89% between weeks 32-52) when feed with a fructose- and sucrose-enriched WD (54). Interestingly, the HCC transcriptomic signature of DIAMOND tumours resembles that of human HCC S1 and S2 subclasses (55). Another recently developed model that progresses to NASH and HCC with an 85% incidence is *MUP-uPA* mice fed with HFD (56). These mice express high amounts of ectopic urokinase plasminogen activator (uPA) in hepatocytes, leading to ER stress and liver damage. Other murine models genetically modified in a diversity of pathways have been reported to be susceptible to NASH and HCC. Among others, these models are associated with dysregulations in receptor tyrosine kinase signalling (hepatic loss of PTEN) (57, 58), lipid catabolism (loss of peroxisomal fatty acyl-CoA oxidase) (59), growth hormone signalling (hepatic loss of STAT5 and glucocorticoid receptor) (60), methionine metabolism (hepatic loss of methionine adenosyltransferase) (61) and hypothalamic regulation of food intake and body weight (total

loss of melanocortin 4 receptor plus HFD) (62). It should be noted that most of these models do not develop overweight and insulin resistance.

Insulin signalling in NASH-driven HCC

Hepatic insulin resistance, *i.e* the incapacity of insulin to adequately suppress hepatic glucose production, is the most common metabolic feature of NAFLD. Some of the underlying mechanisms have been identified and involve the direct and indirect inhibition of insulin-stimulated pathways by pro-inflammatory cytokines (such as TNF- α and IL-6), metabolite intermediates (such as lipids and amino acids), and ER and oxidative stresses (63, 64). It has been recently reported that hyperinsulinemia observed in the course of insulin resistance correlates with impaired hepatic clearance of insulin in patients with NAFLD (65), the liver being the main tissue involved in the degradation of circulating insulin.

Hyperinsulinemia has been identified as a risk factor for HCC development in cirrhotic patients (66-68) as well as in the general population (69). Because hepatic insulin resistance is a major feature of NASH, linking hyperinsulinemia to liver carcinogenesis may seem paradoxical. To resolve this paradox, it has been proposed that pathways may have differential sensitivity to the hormone, some of them remaining insulin-sensitive when they are exposed to higher than normal levels of insulin. This concept stems from the fact that in NAFLD, hepatic *de novo* lipogenesis (DNL) and TG accumulation (physiologically stimulated by insulin) remain activated and are associated with liver steatosis while insulin fails to suppress gluconeogenesis (70). Consistent with this hypothesis, gene expression of gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase catalytic subunit (G6PC) as well as those of the main cytoplasmic insulin receptor substrates (IRS), IRS-1 and IRS-2, are increased in liver samples from patients with NAFLD (71-73). In addition, it appears that mTORC1 lies at a bifurcation of insulin signalling pathways upstream of DNL and gluconeogenesis. mTORC1 is required for insulin-induced upregulation of DNL but not inhibition of gluconeogenesis (74). Of particular note, insulin receptor (IR) signalling is required for the establishment of NAFLD since obese mice with hepatic loss of IR or IRS-1 exhibit marked insulin resistance but lack hepatic steatosis under HFD (75, 76).

During NAFLD, hepatocytes that have escaped death are exposed to intense selection pressure imposed by inflammation, hyperglycemia, oxidative stress and high circulating levels of FFAs. The

maintenance of some insulin signalling pathways could provide selective advantages for premalignant hepatocytes (77). Consistently, the insulin signalling pathway is activated in human HCC due to the overexpression of signalling components or the loss of negative regulators. We previously reported that IR-A (fetal IR isoform) is upregulated to the detriment of IR-B (adult isoform) in HCC due to deregulation of IR pre-mRNA alternative splicing (78). Interestingly, perturbation of IR-A/IR-B expression ratio has been recently reported in livers from patients with NAFLD (73) as well as in experimental murine models of NAFLD (79). IR-A binds not only insulin, but also proinsulin and insulin-like growth factor-II (IGF-II) with high affinity. Proinsulin is present at high levels in the plasma from insulin resistant patients (80). IGF-II is a growth peptide produced by the fetal liver and early after birth, which may be re-expressed in HCC (81). A high IR-A/IR-B ratio is associated with the presence of aggressiveness markers in human tumours and with reduced patient survival after curative hepatectomy (82). In addition, ectopic overexpression of IR-A in human HCC cell lines promotes a migratory/invasive phenotype together with stem/progenitor cell features (82). The expression of IR substrates, IRS-1 and IRS-2, is also frequently increased in HCC compared to adjacent non-tumour liver tissue (83, 84). Loss of hepatic IRS-1 prevents NAFLD progression to HCC in mouse (76). Finally, it has been reported that the loss of different negative regulators of insulin signalling such as SOCS proteins (85-87), GRB14 (88) and PTEN (89) is also frequently observed in human HCC.

Endoplasmic reticulum (ER) stress in NASH-driven HCC

ER is a specialized subcellular compartment involved in folding and quality control of secretory and membrane proteins, calcium homeostasis and lipid synthesis. By regulating proteostasis and lipostasis, ER plays a predominant role in the maintenance of the physiological regulation of hepatic metabolism. However, ectopic accumulation of TG in hepatocytes as observed in NAFLD is often associated with disruption of ER homeostasis promoting ER stress. In case of severe or persistent ER stress, the unfolded protein response (UPR) behaving as an adaptive signalling pathway triggered to restore ER proteostasis can cause steatosis aggravation, insulin resistance, inflammation, inflammasome activation and ultimately hepatocytes death (90). Consistently, markers of ER stress and UPR have been detected in liver tissues from patients and mice with NASH (91-93).

The role of ER stress signalling in HCC development remains to be defined in the context of NAFLD. Using *MUP-uPA* mice fed with HFD, it has been shown that ER stress increases lipogenesis,

oxidative stress, and lipotoxic hepatocyte death. A major consequence of these effects is the induction of inflammatory TNF α -producing macrophages that contribute directly to HCC growth in this model (56). More recently, the same group has identified the non-apoptotic caspase-2 as a key signalling component which expression is induced by ER stress in *MUP-uPA* mouse livers (94). Indeed, *Casp2*^{-/-} *MUP-uPA* mice did not harbour steatosis but exhibited less hepatocyte injury and macrophage infiltration than *MUP-uPA* mice. Caspase-2 promotes the activation of sterol regulatory element-binding protein 1 and 2 (SREBP1/2) which control transcriptional programs leading to TG and hepatic-free cholesterol accumulation, respectively. Consistent with these findings, the expressions of caspase-2, SREBP1, SREBP2 are increased in liver samples from patients with NASH (94, 95).

Of note, murine and human HCC present dysregulation of ER stress and UPR signalling pathways (96-98). While chronic ER stress contributes to steatosis and hepatocyte death in NASH, it is possible that hepatocyte transformation together with other stressors (such as hypoxia, nutrient deprivation, metabolic stress) could alter UPR and prevent ER stress-induced apoptosis that might contribute to cancer cell aggressiveness (99).

Microbiota in NASH-driven HCC

The gut microbiome comprises all of the microorganisms in the digestive tract (over 15,000 species and their million of genes). Dysbiosis, *i.e* imbalance of bacteria found in the gut, can prevent the gut immunologic barrier from functioning properly. Dysbiosis has been associated with the development of chronic metabolic diseases, such as insulin resistance, diabetes, cardiovascular disease, and obesity (100, 101) and linked to inflammation, altered energy homeostasis and choline and bile acid metabolisms, all of them thought important to NAFLD pathogenesis (102).

Consistent with animal data, several human studies have demonstrated that the Firmicutes/Bacteroidetes ratio is increased in obese people compared to lean people, and tend to decrease with weight loss (103) It has been also recently noted that patients with NASH have an altered gut microbiome (104, 105). For example, Proteobacteria, Enterobacteriaceae and Escherichia (at phylum family and genus levels, respectively) were found to be significantly elevated in children with NASH, compared with healthy or obese subjects (105). Another study revealed a reduction in Bacteroidetes in patients with NASH compared to subjects with simple steatosis and healthy individuals, which may facilitate the growth of other bacteria species with increased energy intake from dietary fat

(104). Experimental models support a role for gut microbiota in NAFLD. Germ-free (GF) C57BL/6J mice gain less weight and do not develop steatosis and insulin resistance when given a HFD compared to conventionalized mice (106, 107). Moreover, dysbiosis driven by NLRP6 and NLRP3 inflammasome deficiency has been reported to exacerbate hepatic steatosis and inflammation in different mouse models (*db/db* mice, mice fed with MCDD or HFD) through influx of Toll-like receptor-4 (TLR-4) and TLR-9 agonists into the portal circulation (108). The gut microbiota plays also an important role in the progression of NASH to cirrhosis and HCC through various routes such as alterations in gut permeability, bile acid and choline metabolisms, endogenous alcohol production, activation of TLR and release of proinflammatory cytokines (109-111).

Endotoxemia. The majority of studies on the gut-liver axis in NAFLD have focused on lipopolysaccharide (LPS), a cell wall component of gram-negative bacteria and one of the most potent inducers of inflammation *via* its binding to TLR-4 in hepatocytes and Kupffer cells. Mean portal vein levels of LPS are increased in NASH through mechanisms involving small intestinal bacterial overgrowth, increased intestinal permeability, tight junction alteration and bacterial translocation (112, 113). This creates a subsequent proinflammatory microenvironment into the liver (110). Chronic LPS injection in a mouse model of HCC using DEN and carbon tetrachloride increases tumour burden while reduction of LPS actions using antibiotic regimen or genetic ablation of TLR-4 prevents excessive tumour growth and multiplicity (114, 115). Consistently, mice housed in GF conditions developed fewer and smaller tumours than mice housed in conventional conditions (115). The innate immune system is also activated and mediated by Kupffer cells and hepatic stellate cells (HSC). TLR activation in Kupffer cells results in IL-1 β production that promotes lipid accumulation and cell death in hepatocytes, causing inflammation and HSC stimulation to produce profibrogenic mediators (116, 117).

Bile acids. Dysbiosis could also promote the development of NASH-associated HCC by modifying bile acid metabolism. Primary bile acids (such as cholic acid and chenodesoxycholic acid) are produced by hepatocytes, metabolized as secondary bile acids (such as deoxycholic acid (DCA) and lithocholic acid) by intestinal bacteria in the small intestine, and are critically important for maintenance of a healthy gut microbiota, balanced lipid and carbohydrate metabolisms, insulin sensitivity and innate immunity. Bile acids are ligands for G protein-coupled bile acid receptor TGR5 and nuclear farnesoid X receptor (FXR) (118). Dysbiosis can result in increased production of secondary bile acids in the small intestine. In turn,

bile acids can modulate the composition of the microbiota. The subsequent increase of intestinal reabsorption and return of secondary bile acids to the liver leads to liver inflammation.

Owing to their lipophilic and detergent properties, bile acids can directly disrupt cell plasma membrane and activate p53 and NF- κ B that stimulate inflammatory and pro-tumorigenic pathways through the transcription of genes coding for TNF α , IL-1 β and IL-6. For example, chronic activation of the IL-6/JAK/STAT3 pathway has been implicated in hepatocyte transformation by promoting cell survival and accumulation of DNA damage (50, 119). The hydrophobicity of DCA causes membrane perturbations that activate protein kinase C and NADPH oxidase, which induce downstream activation of NF- κ B. Such membrane perturbations also facilitate the release of arachidonic acid, a pro-inflammatory mediator involved in the synthesis of prostaglandins and leukotriens, which promote ROS production and DNA damage in hepatocytes (118, 120).

In response to a stress, senescence can occur in normal cells which develop a secretory profile composed mainly in inflammatory cytokines, chemokines and proteases. This typical signature is called senescence-associated secretory profile (SASP). The senescence secretome has been proposed to contribute to obesity-associated liver cancer. In diet- and genetically-induced obese mice, increased enterohepatic circulation of DCA provokes SASP in HSC, which leads to the production of proinflammatory cytokines and tumour-promoting factors in the liver that facilitate HCC development upon exposure to the chemical carcinogen dimethylbenzanthracene (121). Importantly, blocking DCA production or depleting gut microbiota with oral antibiotics prevent HCC development and decreases senescent HSC. A reduction in HCC burden is also observed when mice lack a SASP inducer or are depleted in senescent HSC. Signs of SASP have been observed in HSC in the area of HCC arising in patients with NASH indicating that this mechanism may contribute to at least certain aspect of obesity-associated HCC in humans as well (121).

Immunity in NASH-driven HCC

NASH is fuelled by a variety of converging mechanisms that sustain chronic necroinflammation. As a matter of fact, in this context of metabolic reprogramming associating metabolic alterations and lipotoxicity, the very versatile and dynamic inflammatory microenvironment strongly favours HCC development. The molecular mechanisms and the functional contribution of hepatic immune effectors

to cancer emergence in a context of NASH are still insufficiently described but are the subject of intense researches.

It is now admitted that obesity generates a status of inflammation that rapidly becomes chronic, aggravating the risk of developing cancers in general (122). Using mouse models fed with specific diets, the local hepatic immune microenvironment was shown as a critical actor sensitizing to hepatocarcinogenesis in the context of NASH (49, 50). It is noteworthy that sterile inflammation which occurs without pathogens, is recognized as an important mechanism of liver injury during NASH setting. Importantly, damage-associated molecular patterns (DAMPs) (e.g. mitochondrial and nuclear DNA, purine nucleotides...) activate hepatic inflammation through the requirement of inflammasome converting DAMPs into pro-inflammatory signals (123). Consequently, the inflammasome-mediated immune response that leads to the maturation and release of both IL-1 and IL-18 (in a caspase 1-dependent manner) provokes liver injury. Additional effects of PAMPs including bacterial products such as LPS and other bacterial metabolites, translocating from the gut, sustain a particular inflammatory flavor in which $\text{TNF}\alpha$ and IL-6 behave as the main players (124) (125). Of note, liver cells that express TLR recognizing DAMPs and PAMPs include Kupffer cells, HSC, biliary epithelial cells and the insufficiently explored liver sinusoidal endothelial cells (LSECs). This large territory of expression of TLR by liver cells emphasizes the intrinsic ability of the liver to deal with inflammation.

Foremost, innate hepatic effectors such as Kupffer cells have been reported, in both animal models and human disease, as the main resident immune cell subset involved in NAFLD initiation and progression (126). Kupffer cells are decreased in numbers in early stages of NASH onset, and this is followed later on by a strong infiltration of bone marrow-derived Ly6C-positive inflammatory monocytes (127). Moreover, as previously discussed, Kupffer cells exposed to LPS activate through TLR-4, NF- κ B, ERK1, P38, JNK and IRF3 pathways resulting in the genesis of a pro-inflammatory microenvironment and accentuating hepatic damage and triggering fibrogenic response by HSC. Importantly, in DEN-induced HCC mouse model, pro-inflammatory activation of Kupffer cells and notably myeloid cell receptor TREM-1 expression was evidenced as a determinant feature in driving HCC development (128, 129). Although resident and infiltrating myeloid cells are extensively studied, molecular mechanisms depicting their precise contributions to NASH-HCC transition are still missing. However, recent data provide evidence that Kupffer cells through their interactions with platelets are crucial in the early triggering of inflammation during NASH (130).

Beside Kupffer cells, Natural Killer T (NKT) cells have been shown to display detrimental effects during NASH in CD-HFD mouse model (49). Indeed, these TCR-invariant lymphocytes recognizing lipid species, bridging the gap between innate and adaptive immunity, have been found to harbour strong abilities to favour lipid storage in hepatocytes through the secretion of LIGHT (a member of TNF family also known as TNFSF14). In concert with NKT cells and under the same regimen, intrahepatic activated CD8⁺ T cells have been found unexpectedly to accentuate liver damage through the production and release of large quantities of interferon γ . These two lymphocyte populations create a permissive microenvironment favouring the emergence of transformed NASH hepatocytes. By contrast, CD4⁺ T cells have been shown to exhibit protective effects in limiting both tumour initiation and tumour progression (131, 132). Indeed, it has been reported that pre-malignant senescent hepatocytes secrete chemo- and cytokines and are subject to immune-mediated clearance which critically depends on an intact CD4⁺ T-cell-mediated adaptive immune response (in an antigen-specific-dependent manner). Therefore, impaired immune surveillance favours the development of HCC evidencing that senescence surveillance is important for tumour suppression *in vivo* (132). Moreover, using other NAFLD and HCC models, the metabolic contexture induced by NASH is demonstrated to severely impact on CD4⁺ T cell functionality leading to their selective depletion over time. This ultimately results in an acceleration of tumour development in liver-specific inducible MYC transgenic mice fed a MCDD (133). Moreover, other subsets of CD4⁺ T cells such Th17 CD4⁺ T cells induce pejorative effects as these cells produce IL-17A consecutively to DNA damage induced by excess nutrient overload (activation of unconventional prefoldin RPB5 interactor (URI)). This results in the recruitment of neutrophils to white adipose tissue mediating insulin resistance, FFA release, TG storage in the liver, and initiating NAFLD (134). In the same study, it has been reported that elevated URI in human HCC samples correlates with elevated expression of IL-17A suggesting that the URI/IL-17A pathway impacts on NASH/HCC transition. As a matter of fact, depending on the subset, CD4⁺ T cells demonstrate opposing behavior in HCC either anti-inflammatory or pro-tumorigenic functions.

Finally, ignored for a long period of time, B cells have been recently investigated during NASH and NASH/HCC transition. Initially, B cells were known to drive CD4⁺ T cell activation in white adipose tissue during obesity and subsequently participate to liver fibrosis in an antibody independent manner. However, circulating IgA levels have been found to correlate with severe fibrosis in patients with NAFLD (135). Further work has recently evidenced, in both humans and mice, that resident IgA⁺-PDL1⁺-cells

(PDL1, Programmed cell death 1 ligand 1) accumulate in NASH livers and behave as strong suppressive immune entities against CD8+ T cells in a context of NASH-induced HCC (53).

Collectively, HCC development in NAFLD is intimately associated with complex changes at the immuno-metabolic interface. Further studies on forthcoming characterized immune subsets of the liver microenvironment such as Mucosal Invariant T Cells recently evidenced as a profibrogenic immune cell population in the liver (136), innate lymphoid cells, NK cells, $\gamma\delta$ T cells, and myeloid/dendritic cells will necessarily bring another immune landscape to improve immunotherapeutic intervention to cure this disarming disease.

Management of HCC in the context of NASH

The management of HCC is based on the Barcelona Liver Clinic Classification (BCLC) according to EASL guidelines (137). This classification has been created and modified during the years considering a population of patients with HCC arising mostly on viral hepatitis and alcohol-related cirrhosis. There are no specific recommendations about the management of NASH-associated HCC. It has to be taken into account that this specific group of HCC is not completely comparable to HCC from other etiologies, especially in terms of cancer stage at diagnosis and treatment allocation (138).

Only 15% of patients with NASH-related HCC are categorized at the earliest stages at diagnosis (stage BCLC 0 or A), mostly because one of the criteria considered by the BCLC classification is the performance status (19). This item is directly influenced by comorbidities such as cardiovascular disease and obesity and therefore is often altered in patients with NASH. Patients in this class are eligible to curative treatment such as surgical resection, percutaneous ablation or liver transplantation. However, therapeutical option is rarely feasible in NAFLD-related HCC. 17% patients with NASH-related HCC are included at diagnosis in stage BCLC B and allocated to transarterial chemo-embolization (TACE) (19). The vast majority of patients with NASH-related HCC (65-75%) is classified as stage BCLC C or D (19). As HCC screening is not recommended for patients with NAFLD/NASH without cirrhosis, these patients are diagnosed at a more advanced stage and age (139). About 50% of patients with NASH-related HCC are at stage BCLC C at time of diagnosis and thus eligible to neither a curative treatment nor a first-line palliative treatment such as TACE. This subclass of patients is considered to be the most heterogeneous because it includes patients with advanced HCC in good clinical condition or with early HCC with comorbidities and a low performance status (138). Patients that are stage BCLC C are generally treated

with sorafenib, a multi-kinase inhibitor that provides a modest gain of survival (nearly 10 weeks) (140, 141). In the two phase-3 clinical trials confirming the efficacy of sorafenib in advanced HCC, the number of patients with NASH-related HCC was low, so the efficacy of this targeted therapy in this specific subset of patients is not clearly demonstrated. Similarly, no data are available regarding the effects of lenvatinib, a more recently validated first-line therapy (142) and those of the two second-line therapies regorafenib (143) and cabozantinib (144) in patients with NASH-related HCC.

The clinical outcome of patients with HCC developed on a NASH liver has been reported to be worse than that of patients with HCC related to HCV or HBV cirrhosis (145), probably due to multiple factors such as a lack of specific screening program leading to patient older age and lower performance status at diagnosis and a higher tumour burden reducing patient access to therapies. In contrast, another study reported a similar survival between patients with NASH- and non-NASH-related HCC while it confirmed the differences in terms of higher age at diagnosis, larger tumour burden and comorbidities between the two groups of patients (146).

Potential molecular strategies in NASH-driven HCC

A better understanding of the pathogenesis of NASH progression to HCC has led to the identification of several potential therapeutic targets.

Metformin. Metformin, an anti-diabetic drug currently used in clinical practice, has been reported to reduce the risk of developing HCC in patients with T2DM (147). Recently, metformin has been investigated in a zebrafish model of NAFLD/NASH-associated HCC. This study suggests that metformin treatment could inhibit HFD-induced cancer progression by reducing non-resolving inflammation and restoring tumour surveillance (148).

ER stress. As previously discussed, ER stress plays a significant role in NASH progression. In this setting, two actors of ER stress, B-cell lymphoma 2 (BCL2)-associated X protein (Bax) inhibitor-1 (BI-1) and caspase-2 have been identified as potential targets. BI-1 is a negative regulator of the inositol-requiring enzyme 1 alpha (IRE1 α) arm of UPR which is a crucial regulator of hepatic lipid metabolism (90). In human NAFLD liver biopsies, an inverse correlation is observed with down-regulated BI-1 expression and up-regulated IRE1 α ribonuclease signalling (149). The hepatoprotective role of BI-1 against NASH has been recently explored in murine models. BI-1 knockdown mice fed with HFD present NASH and T2DM together with NLRP3 inflammasome activation, hepatocyte injury, fibrosis, and

dysregulated lipid homeostasis. The pharmacological inhibition of IRE1 α ribonuclease activity with the small molecules, STF-083010 or 4 μ 8c, improves glucose tolerance and rescue BI-1^{-/-} mice from NASH. Caspase-2 is activated during ER stress and promotes the production of SREBPs. Caspase-2 ablation or its pharmacological inhibition in mice prevents diet-induced steatosis and NASH progression (94).

FXR agonists. FXR plays an important role in the physiological regulation of bile acid metabolism in the liver. Loss of FXR signaling is likely associated with hepatocarcinogenesis but the contribution of tissue-specific FXR deficiency remains unclear. Thus, Fxr-null mice are prone to develop spontaneous HCC with age (150, 151) and FXR has been found to be downregulated in human HCC specimens and correlated with more aggressive cancer features (152). Several studies have reported that FXR activation with agonists such as chenodeoxycholic acid and obeticholic acid could reduce liver cancer progression in experimental models mice (153, 154). However, a recent study has reported that FXR agonists enhance transforming growth factor- β -induced epithelial–mesenchymal transition (EMT) morphologic changes in human HCC cell lines (155). As the expression of EMT markers has been associated with tumour aggressiveness, further *in vivo* evaluation of FXR agonists should be performed in experimental models of HCC to investigate whether they may have such detrimental effects. Several FXR agonists have reached late-phase clinical trials for treatment of NASH (obeticholic acid, GS-9674, LY2562175...) which also places them as a preventive approach for HCC (156).

Cholesterol absorption. High levels of cholesterol are linked to an increased production of vascular endothelial growth factor (VEGF) in several models, a pivotal factor in the development of HCC (157). Ezetimibe, an inhibitor of cholesterol absorption, has been investigated in PTEN-deficient mice fed with HFD which show hypercholesterolemia together with increased VEGF levels. In this model, ezetimibe reduces the development of liver tumours, lowering cholesterol and reducing liver inflammation, fibrosis and angiogenesis (158).

De novo lipogenesis. DNL is exacerbated during NASH and HCC development. The synthesis of new membranes and specific lipids is essential for cancer cell growth and survival. The expression of key lipogenic enzymes such as acetyl-CoA carboxylase 1 (ACC) is progressively induced from nontumoral liver tissue towards HCC and correlates with clinical aggressiveness and poor survival (159-161). The abrogation of lipid synthesis through the silencing of lipogenic enzymes or transcription factors with RNA interference reduces proliferation and survival in human HCC cell lines (160, 162). ND-654, a liver-

specific inhibitor of ACC inhibits hepatic DNL and tumour development in a rat model of sequential cirrhosis and HCC when used alone or in combination with sorafenib (163).

Peroxisome proliferator-activated receptor- γ coactivators. In the liver, the activity of peroxisome proliferator-activated receptor (PPAR) α and β is under tight control of PPAR- γ coactivators-1 (PGC-1) α and β . PGC-1 β is a critical regulator of hepatic mitochondrial functions such as oxidative metabolism, mitochondrial biogenesis, antioxidant responses and DNL (164). Using hepatic-specific PGC-1 β -overexpressing and PGC-1 β knockout mice treated with DEN to promote liver carcinogenesis, it has been reported that PGC-1 β -overexpressing mice show acceleration of HCC development while PGC-1 β knockout mice are protected (165). Promotion of liver cancer in PGC-1 β -overexpressing mice is associated with increased ROS scavenger activity and lipogenic gene expression. Until recently, the PGC-1 pathway has been considered as difficult to be selectively targeted by drug design. The small synthetic molecule SR-18292 which selectively inhibits PGC-1 α restores glucose homeostasis in diet- and genetically-induced obese mice (166). Considering these data, PGC-1 family is an attractive target for future drug development in NASH-related HCC.

Cell cycle-related kinase. Another potential molecular target recently identified in HCC development in the context of obesity and NASH is cell cycle-related kinase (CCRK), an androgen receptor (AR)-driven oncogene. Indeed, the components of the STAT3-AR-CCRK-mTORC1 pathway are overexpressed in human NASH-associated HCC and the hepatic knock-down of CCRK in mice fed with HFD abrogates steatosis, insulin resistance, and HCC development. This pathway seems to exert a double role by both promoting tumour growth and creating a pro-tumourigenic microenvironment *via* its immune suppressive action (167).

Platelet activation. Mean platelet volume, a surrogate marker for platelet activation, has been reported to be increased in biopsies from NASH patients compared to healthy subjects (168). Moreover, it has been reported that activated platelets could contribute to the homing of many effector cells of inflammation (NKT, cytotoxic CD8⁺ T lymphocytes) in models of viral hepatitis. By using multiple mouse models of NASH, it has been confirmed that platelet number, activation and aggregation together with the recruitment of inflammatory cells are increased in NASH livers but not in steatotic and insulin resistance states (130). Platelet homing and activation is linked to the expression of hyaluronic acid in the liver, the platelet receptor subunit glycoprotein 1b alpha (GPIb α), a substrate for protein disulfide isomerase known to have important functions in thrombosis and the presence of Kupffer cells. Blocking

GPIIb/IIIa with either antibodies or transgenic mice lacking functional GPIIb/IIIa as well as antiplatelet therapy with aspirin/clopidogrel/ticagrelor but not with nonsteroidal anti-inflammatory drug inhibits the development of NASH and subsequent HCC (130).

Manipulating immune responses. The landscape of medical therapy has been impressively completed by the arrival of very encouraging studies aiming at manipulating the immune system. Indeed, through the use of immunotherapy targeting immune check point inhibitors (ICI) expressed by a variety of immune cells, recent data provided very promising results, showing that up to 20% of patients with HCC - including all aetiologies - respond to these drugs, with survival benefit markedly exceeding that seen for kinase inhibitors (169, 170). The evaluation of the combination of several immune blockers on HCC is currently under investigation. But of importance, in this novel era of treatments, one should consider that immunotherapy is not reduced to the use of ICI but should include other immunomodulators of the immune response and found other pertinent strategies (CAR T cells, vaccination using peptides or DNA, cytokine/chemokines antibody blockade, adoptive immune cell transfer...) to accentuate or circumvent the function of the immune population targeted during the course of the disease. As an emerging topic, the role of exercise in modulating immune response and promoting an anticancer immune microenvironment associating changing in gut microbiota is an exciting field of intense and promising researches for HCC patients with metabolic syndrome (171, 172).

Concluding remarks

The increasing prevalence of obesity and D2TM is influencing the epidemiology of NASH and NASH-related HCC. The most dramatic increase of NAFLD-related HCC is currently seen in high socio-economical countries. However, changes in eating habits and increased sedentary lifestyles worldwide has now spread to every country in the world and the incidence of this fatal cancer will continue to rise globally. Accumulated evidence makes NASH the most common causes of HCC in upcoming years. Diagnosis of NASH-related HCC frequently occurs at advanced stages of tumor development, rendering curative options futile. This calls for the development of alternate guidelines for HCC surveillance and early diagnosis in at-risk patients. To achieve this objective, the identification of biomarkers that allow patients with NASH to be stratified according to their risk of developing HCC and early detection of tumours will be an important issue. Laboratory findings from *in vivo* mouse models have increased our

understanding of the pathogenesis of NASH and its progression towards HCC. Many complex processes implicating organ-organ cross-talks and interactions between liver parenchymal and nonparenchymal cells have been unravelled. No hierarchy of importance for these pathways in HCC emergence have been established yet. Some of the available mouse models of NASH progression towards HCC have inherent specific disadvantages and do not recreate the disease condition in humans (use of toxins, mild fibrosis, lack of validation against human...). Therefore, there remains a need in developing new pre-clinical models that will better recapitulate the human pathology. Medications to halt the progression of NASH are also urgently warranted and several approaches are currently under investigation. Combination treatments targeting different pathogenic “hits” could be useful to reduce risk of malignant transformation .

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Legend to Figure 1. Schematic conceptualizing the natural history of HCC in a NAFLD context

Obesity and T2DM contribute to the development of insulin resistance and hepatic steatosis. In this context of metabolic imbalance and together with genetic predisposition (such as *PNPLA3* polymorphism), multiple parallel hits coming from adipose tissue (free fatty acids (FFAs), glycerol, pro-inflammatory cytokines IL-6 and TNF- α) and gut (lipopolysaccharide (LPS), biliary acids (BA)) converge to the liver and initiate tissue injury. Lipotoxicity, unresolved endoplasmic reticulum (ER) stress and excessive oxidative (Ox) stress are proposed as the main culprits in driving the death of steatotic hepatocytes and the release of damage-associated molecular patterns (DAMPs) that, together with pathogen-associated molecular patterns (PAMPs), initiate a vicious cycle of pro-inflammatory response *via* toll-like receptors (TLRs). Direct recruitment of Kupffer cells and other components of the innate immune response occurs with activation of the NLRP3 inflammasome and the coordinated release of pro-inflammatory and pro-fibrogenic cytokines and chemokines. Hepatic stellate cells (HSCs) are subsequently activated to produce extracellular matrix leading to progressive fibrosis and cirrhosis. The establishment of chronic necroinflammation initiates an adaptive immune response which ultimately leads to cellular stress, DNA damage, epigenetic modifications, and chromosomal aberrations in liver cells. At the same time, exhaustion and tolerance of adaptive immune cells diminish the important role of these cells in the detection and elimination of transformed cells.

Figure 1