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Abbreviations: BMI, body mass index; CI, confidence interval; CLD, chronic liver disease; EPIC, european prospective investigation into cancer and nutrition; FOXO1, Forkhead box O1; G6PC, glucose 6-phosphatase catalytic subunit ; HBV, hepatitis B virus; HCC, hepatocellular carcinoma; HCV, hepatitis C virus; HOMA, homeostasis model assessment; IGF, insulin-like growth factor; IL, interleukin; IR, insulin receptor; IRS, insulin receptor substrate; MAPK, mitogen-activated protein kinase; mTORC, mammalian target of rapamycin complex; NAFLD, non-alcoholic fatty liver disease; NASH non-alcoholic steatohepatitis; PEPCK, phosphoenolpyruvate carboxykinase ; PI3K, phosphatidylinositol 3-kinase; RR, relative risk; SOCS, suppressor of cytokine signalling; TG, triglycerides; TNF, tumour necrosis factor; VEGF, vascular endothelial growth factor.

Conflict of interest: none

Key points:

- Hyperinsulinemia compensatory to insulin resistance is common in the course of chronic liver diseases, irrespective of etiology.

- Insulinemia is an independent risk factor for HCC.

- In NAFLD, clinical and experimental evidence reveal that hepatic insulin resistance is partial; some pathways remain insulin-sensitive.

- HCC tumors exhibit major dysregulations in insulin-dependent pathways.

Abstract

Hepatocellular carcinoma (HCC) is the fifth most prevalent cancer and is one of the leading causes of cancer-related death. The risk factors for HCC include cirrhosis, chronic viral hepatitis, heavy alcohol intake and metabolic diseases such as obesity, type 2 diabetes and metabolic syndrome. Insulin resistance is a common denominator of all of these conditions and is tethered to hyperinsulinemia. Here, we give an overview of the recent advances linking hyperinsulinemia to HCC development and progression. In particular, we summarize the underlying causes of hyperinsulinemia in the setting of chronic liver diseases. We present epidemiological evidence linking metabolic diseases to HCC risk and HCC-related mortality, as well as the pathogenic cellular and molecular mechanisms explaining this relation. A better understanding of the mechanisms by which insulin participates in HCC biology might ultimately provide novel opportunities for prevention and treatment.

Introduction

Hepatocellular carcinoma (HCC) is a primary malignancy of the liver arising from mature hepatocytes or hepatic progenitors. HCC is the fifth most common cancer worldwide, with more than 800,000 new cases annually and the second cause of cancer deaths. HCC is overwhelmingly related to chronic liver diseases (CLDs) associated with persistent inflammation, fibrosis and cirrhosis. Development of HCC in the cirrhotic liver is a stepwise process, which follows a dysplasia-carcinoma sequence that takes several decades to evolve. Chronic hepatitis B (HBV) and C (HCV) virus infections are the leading risk factors of HCC worldwide, especially in Southeast Asia and Sub-Saharan Africa. In the US and Western countries where HCC incidence is rapidly rising, alcoholic cirrhosis and non-alcoholic fatty liver disease (NAFLD) are also important risk factors. HCC is a tumour with poor outcomes and limited therapeutic options (reviewed in [1-3]).

The liver plays a crucial role in the control of glucose metabolism by insulin and CLDs - irrespective of etiology – are prone to be associated with hepatic insulin resistance. Insulin resistance is defined as a refractory state of the liver to the negative regulatory effect of insulin on glucose production. Rise in circulating insulin levels is frequently associated with CLDs, resulting both from the impairment of hepatic insulin degradation and from the activation of insulin secretion by pancreatic beta-cells, a compensatory mechanism allowing to maintain euglycaemia in the early course of insulin resistant states. The relationship linking hyperinsulinemia and HCC is also bi-directional. Indeed, hyperinsulinemia resulting from insulin resistance, is a key feature of obesity and type 2 diabetes, which have emerged as important contributing diseases to HCC these last years. These metabolic diseases are closely associated to the development of non-alcoholic steatohepatitis (NASH), a severe

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form of NAFLD defined histologically as the coexistence of hepatic fat accumulation and inflammatory changes and which is a risk factor of HCC (reviewed in [3-5]).

Apart from diabetes, which is a risk factor for several cancers, prospective epidemiological studies have identified high circulating endogenous levels of insulin in non-diabetic subjects, as a strong and independent risk factor for the development of solid tumours such as breast, colorectal and pancreatic cancers [6-8]. In addition, hyperinsulinemia has been associated with more aggressive cancer phenotypes and poor prognosis [9-12]. As the prevalence of obesity and diabetes has dramatically increased worldwide during the last decades, cancers associated with hyperinsulinemia may drastically increase in the coming years.

Accumulating evidence suggests that there is a causative link between hyperinsulinemia and HCC development and progression. While the activation of insulin-dependent signalling pathways is certainly not sufficient to initiate tumorigenesis on its own, hepatocytes may develop adaptative insulin-dependent mechanisms to proliferate and survive during CLDs, which may in turn promote premalignant transformation and tumour growth. In addition, insulin may foster a microenvironment milieu favourable to the propagation of premalignant and malignant cells.

This review aims at summarizing key evidence linking hyperinsulinemia and insulin-dependent signalling pathways to the pathogenesis and the progression of HCC. We present recent epidemiological and clinical evidence supporting this association. We also delineate the potential underlying mechanisms by highlighting studies derived from human liver tissues and murine models.

Physiological insulin signalling in the hepatocyte

Insulin is a major anabolic hormone produced by the pancreas primarily in response to glycemia elevations. Insulin plays a central role in the postprandial storage and utilisation of nutrients from food intake by regulating metabolism of carbohydrates, lipids and proteins in liver, skeletal muscle and adipose tissue. In the liver, insulin acts on hepatocytes to inhibit gluconeogenesis and to stimulate glycolysis, glucose storage as glycogen, protein synthesis and lipogenesis (reviewed in [13]). Insulin also induces cell swelling in hepatocytes that is critical for the stimulation of glycogen and protein synthesis [14]. Apart from its metabolic effects, insulin is also a prominent growth factor for hepatocytes favouring their division through the G_1/S and G_2/M transitions and their survival. Insulin also controls late mitosis progression and terminal differentiation in hepatocytes by regulating cytokinesis and tetrapolyploidization [15].

Insulin mediates its biological effects through binding to a heterotetrameric $\alpha_2\beta_2$ receptor tyrosine kinase expressed at the plasma membrane of hepatocytes. Two isoforms of insulin receptor (IR) arise from alternative splicing of IR pre-mRNA, resulting from inclusion (isoform IR-B) or skipping (isoform IR-A) of exon 11 encoding 12 amino acids located at the carboxyl-terminus of the extracellular a-subunit. IR pre-mRNA splicing is developmentally regulated and IR-A is predominantly expressed in embryo and fetal tissues including the fetal liver [16]. IR-A becomes less expressed as differentiation progresses and adult hepatocytes exclusively express the IR-B isoform. Binding of insulin to IR-B leads to receptor tyrosine autophosphorylation and tyrosine phosphorylation of cytosolic substrates (including insulin receptor substrate (IRS)-1, IRS-2 and the adapter protein Shc), which activate a complex network of intracellular pathways including the two well-studied phosphatidylinositol 3-kinase

(PI3K)-AKT and Ras/mitogen-activated protein kinase (MAPK) pathways (reviewed in [17]) (**Figure 1**). AKT (also known as protein kinase B) is central for the ability of insulin to regulate metabolism and multiple targets of AKT are involved in insulin action. Insulin stimulation of AKT activity through 3-phosphoinositide-dependent protein kinase 1 (PDK-1) and mammalian target of rapamycin complex 2 (mTORC2)-dependent-mechanisms leads to the inhibitory phosphorylation of the transcription factor Forkhead box O 1 (FOXO1). This results in the blockage of FOXO1 transcriptional activity on gluconeogenic genes such as those encoding phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase catalytic subunit (G6PC). The ability of insulin to stimulate hepatic lipogenesis in the liver depends upon the induction of the transcription factor sterol regulatory element-binding protein 1c (SREBP-1c), which involves AKT- and mTORC1-dependent mechanisms. Interestingly, an insulin-stimulated non-canonical AKT-independent pathway involved in the regulation of hepatic glucose production has recently been identified by Lu and colleagues in triple AKT1/AKT2/FOXO1 knock-out mice [18].

Chronic liver diseases, insulin resistance and compensatory hyperinsulinemia

The liver plays a pivotal role in the control of glucose metabolism by insulin. Therefore, CLDs, which impair hepatic functions are frequently associated with changes in carbohydrate metabolism. In the diseased liver, insulin has a decreased ability to suppress hepatic gluconeogenesis and hepatic insulin degradation is impaired. Both mechanisms favor hyperinsulinemia, which initially compensates insulin resistance, but gradually associates with hyperglycemia. It is commonly accepted that insulin resistance is a key contributing factor to the pathogenesis and progression of CLDs (**Figure 2**).

Cirrhosis. Cirrhosis consists in a fibrotic nodular transformation of the liver and is a long-term consequence of many different CLDs such as viral hepatitis, iron overload disease, NAFLD and alcohol abuse. Cirrhosis reduces the mass of functional hepatocytes and leads to portosystemic shunting. Whatever its underlying cause, cirrhosis predisposes to HCC and is considered as a preneoplasic condition. The vast majority of HCC worldwide develops in patients with pre-existing cirrhosis [19]. Cirrhosis is also a diabetogenic condition and diabetes mellitus secondary to cirrhosis is sometimes referred to as « hepatogenous diabetes ». The prevalence of insulin resistance, hyperinsulinemia and glucose intolerance among patients with cirrhosis is by far higher than in subjects without liver diseases. Sixty to eighty percent of patients with cirrhosis display hyperinsulinemia and/or glucose tolerance abnormalities and up to 20% develop overt diabetes. In addition, insulin resistance is an established risk factor for disease progression and reduced survival in patients with cirrhosis (reviewed in [20]). Hyperinsulinemia associated with cirrhosis could be linked not only to increased insulin secretion from pancreatic islets but also to a decreased hepatic clearance of insulin [21]. The liver is the main tissue involved in the clearance of circulating insulin. Plasma insulin half-life is 4-6 minutes and nearly 70% of the portovenous insulin is removed from the circulation after the first passage through the liver [22]. Due to portosystemic shunting, reduced hepatic function and exacerbated hepatocellular insulin resistance, insulin clearance from the circulation that occurs mainly *via* receptor-mediated endocytosis, is reduced in patients with cirrhosis.

HCV infection. Chronic HCV infection is associated with the highest HCC incidence in persons with cirrhosis (4-5% yearly cumulative incidence [19, 23]). Epidemiological studies have suggested a link between HCV infection and diabetes, implying

hepatitis C as a metabolic disease. Patients with chronic HCV infection are at greater risk of developing insulin resistance and diabetes at early stage of liver disease, even in the absence of hepatic fibrosis compared with non-infected individuals or patients with HBV infection (RR: ~1.7; reviewed in [24, 25]). The *ex vivo* analysis of liver biopsies from HCV patients, the transfection of HCC cell lines with HCV core proteins and the use of transgenic mice expressing HCV core proteins have revealed that HCV core proteins can directly interfere with hepatocyte intracellular insulin signalling. HCV core proteins promote inhibitory phosphorylation of IRS on serine residues and IRS proteasomal degradation through the up-regulation of suppressor of cytokine signalling-3 (SOCS-3) [26-29]. HCV infection may also favour insulin resistance through indirect mechanisms involving the production of proinflammatory cytokines such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) together with steatosis (an accumulation of triglycerides (TG) as fat droplets within the cytoplasm of hepatocytes) and mitochondrial dysfunction, all factors contributing to alter insulin signalling [25, 30].

Nevertheless, the concept of HCV as a diabetogenic virus *per se* has been challenged recently by Everhart and colleagues [31]. These authors did not find any association between HCV infection and diabetes or insulin resistance in a large U.S population of 15,128 adults. Rather, they identified a link between elevated liver enzymes and diabetes/insulin resistance, regardless of the HCV status. This suggests that the severity of the underlying liver disease is a confounding factor that may have been underestimated in previous studies.

NAFLD. NAFLD is a spectrum of liver abnormalities ranging from simple and benign steatosis to NASH, a steatohepatitis that includes necroinflammation changes and variable degrees of fibrosis in the absence of alcohol abuse. NAFLD is considered to

be the hepatic manifestation of metabolic diseases and is related to the presence of obesity, diabetes and metabolic syndrome. Ectopic lipid accumulation in the liver develops when the rate of hepatic TG synthesis (*via* increased fatty acid uptake and esterification into TG and *de novo* synthesis) exceeds the rate of TG catabolism (*via* β-oxidation of fatty acids and export as very low density lipoproteins). It is estimated that up to 70% of patients with type 2 diabetes and up to 90% of obese patients have some degree of fatty liver disease. Approximatively 20% of all cases of NAFLD present as steatohepatitis (reviewed in [3, 5, 32]. The exact prevalence of HCC in patients with NASH-related cirrhosis remains unknown. A study from the US found that the yearly cumulative incidence of HCC is 2.6% in patients with NASH-related cirrhosis, compared with 4.0% in patients with HCV-driven cirrhosis [23]. It has been reported that a significant number of patients with NAFLD-related HCC (more than one third) have no extensive fibrosis at presentation (reviewed in [5]). This suggests that the chronological sequence fibrosis/cirrhosis/HCC is not the sole carcinogenic pathway in the setting of NAFLD. Metabolic dysregulations observed during NASH could play an important role in promoting HCC.

Insulin resistance and hyperinsulinemia are the most common metabolic features of NAFLD. Hyperinsulinemia in NAFLD correlates with impaired hepatic clearance of insulin [33]. The pathophysiological mechanisms of insulin resistance in the setting of NAFLD are multifactorial and have been reviewed recently [3, 30, 34, 35]. Briefly, they include low-grade systemic inflammation (*via* the release of pro-inflammatory cytokines such as TNF-α and IL-6), imbalance in adipokine secretion, lipotoxicity (*i.e.* cellular dysfunctions linked to the accumulation of lipid intermediates such as diacylglycerols), modifications in the gut microbiota, mitochondrial defects and oxidative stress. These mechanisms contribute to impair insulin signalling at

receptor and post-receptor levels by activating a variety of serine/threonine kinases (including c-Jun amino-terminal kinase, inhibitor kB kinase-β, conventional and novel protein kinases C, mTORC1/S6 kinase and MAPKs which promote inhibitory phosphorylation of IR and of its main substrates IRS-1 and IRS-2). The activation of transmembrane and cytosolic phosphoprotein phosphatases which are negative regulators of insulin action such as PP2A, PTP1B and SHP2 has also been reported in insulin resistant states (reviewed in [17, 36]). Proinflammatory cytokines contribute to cellular insulin resistance by inducing the expression of SOCS-1 and -3 proteins which prevent IRS-1 and IRS-2 tyrosine phosphorylation and/or promote IRS degradation (reviewed in [24, 37]).

Epidemiological evidence linking diabetes, obesity and metabolic syndrome to HCC development and progression

Diabetes, obesity and metabolic syndrome have been repeatedly associated with increased incidence for several cancers. Substantial epidemiological evidence has revealed that the incidence and prognosis of HCC are also significantly affected by these conditions, supporting a physiopathological link – probably through NAFLD - between metabolic factors and liver cancer.

Type 2 diabetes. A positive association between diabetes and HCC has been reported in multiple observational studies from Asia, Europe and North America, independently of the cause underlying cirrhosis and/or CLD. Recent meta-analyses conducted from prospective studies concluded that the diabetic status is associated with a more than two-fold higher risk of HCC [38-40]. A prospective study based on the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort (363,426 apparently healthy men and women recruited from 10 European countries

between 1992 and 2000 ; mean follow-up time of 8.5 years) also supports this conclusion [41]. Moreover, the prospective analysis of a U.S. cohort including over one million subjects enrolled in the Cancer Prevention Study-II from 1982 to 2008 revealed that diabetes is associated with a higher risk of death from HCC (RR: 1.40; 95% CI [1.05-1.86]) [42]. The association between diabetes and HCC was linked with diabetes duration in some [43, 44] but not all studies [41, 45, 46]. There is a synergistic interaction between diabetes and other HCC risk factors such as virus and alcohol [47-49]. In addition, diabetes worsens HCC prognosis and impacts the surgical outcome of patients with cirrhosis after liver resection and transplantation [50].

Obesity. Obesity (defined as body mass index (BMI) $\geq$ 30 kg/m^2) is a well-known risk factor for insulin resistance. Obesity, independently of type 2 diabetes, is implicated in initiation and progression of cancer at multiple sites including the liver [51, 52]. Thus, in a recent meta-analysis of 26 prospective studies conducted in Europe, US and Asia (25,337 HCC cases published between 1994 and 2011), it has been shown that the risk of HCC is about twice higher among obese patients - specially males - than in normal-weight individuals (RR: 1.83; 95% CI: 1.59-2.11) [53]. This positive association is independent of history of diabetes, viral infections and alcohol abuse. A prospective study based on the EPIC cohort confirmed the association between obesity - specially abdominal obesity, more specifically linked to insulin resistance - and risk of HCC [54]. Obesity is also the leading risk factor of HCC-related cancer deaths in middle-aged men in the USA. Thus, a large prospective study conducted in more than 900,000 US citizens followed up for 16 years reported that in obese patients, the risk of mortality from HCC was much higher (RR: 4.52, 95% CI: 2.94-6.94 for men; RR: 1.68, 95% CI: 0.93-3.05 for women) than that from other cancers

[55]. Obesity also increases the risk of HCC in populations already at high risk such as those with cirrhosis and viral hepatitis [48, 56, 57]. Obesity predicts a poor outcome after surgery and doubles HCC recurrence [58, 59].

Metabolic syndrome. The metabolic syndrome is a condition related to insulin resistance, defined as the association of central obesity with two or more other cardiovascular and/or diabetes risk factors including increased levels of plasma TG or glucose, reduced HDL-cholesterol and raised blood pressure [60]. In agreement with data on diabetes and obesity, a recent meta-analysis of 10 case series and longitudinal studies published through October 2011 concluded that the presence of metabolic syndrome is associated with the occurrence of liver cancer in men (RR: 1.43, 95% CI: 1.23-1.65) [61]. Two more recent studies performed on 3,649 HCC cases in the US confirmed that a pre-existing metabolic syndrome confers a statistically increased risk for HCC that is independent of other risk factors (RR: 2.13, 95% CI: 1.96-2.31 [62]; RR: 1.97, 95% CI: 1.03-3.79 [63]).

The increased amount of fat by itself is unlikely to be the only factor linking obesity to insulin resistance in metabolic syndrome. Several studies have shown that the limitation of adipose tissue expandability is a major driving factor of insulin resistance and inflammation (reviewed in [64]). Adipose tissue expansion is altered as a result of intrinsic limits in preadipocyte formation (mainly due to genetic factors and/or aging) and/or from altered maintenance of mature adipocytes (linked to fat inflammation or fibrosis among other factors). This increases lipid flux and induces ectopic lipid deposition in the liver. In addition, in the metabolic syndrome, the predominant enlargement of intra-abdominal fat, which is physiologically more insulin resistant than subcutaneous fat, further favours increased lipolysis and exposition of the liver to free fatty acids and pro-inflammatory cytokines.

Insulin resistance markers, homeostasis model assessment (HOMA) index and plasma insulin levels, are predictors of HCC occurrence

As insulin resistance is a common metabolic disorder occuring in obesity, diabetes and metabolic syndrome, it is suspected that hyperinsulinemia is related to HCC. In favor, the HOMA index, a marker of insulin resistance which integrates both fasting glycemia and insulinemia, has been shown to be predictive of HCC occurrence and liver-related death or tranplantation in HCV patients with cirrhosis, independently of BMI [65-67]. Elevated fasting insulin, which is inversely related to insulin sensitivity, has been shown to be an independent risk factor for HCC in a prospective cohort of 2,903 male HBV carriers from Taiwan [68]. More recently, baseline serum levels of C-peptide (released from pro-insulin during beta-cell insulin secretion) have been found to be associated with a higher risk of HCC in the general population issued from the prospective EPIC cohort, independently of obesity parameters and other established liver cancer risk factors [69].

Diabetes treatments and their impact on HCC development

Several observational studies have suggested that some anti-diabetic medications could modify the risk of HCC in patients with diabetes mellitus. Two recent meta-analyses concluded that the biguanide metformin, which is the first-line drug for the treatment of type 2 diabetes, is associated with a diminished risk of HCC among patients with diabetes [70, 71]. Metformin exerts its antidiabetic effects mostly by decreasing hepatic gluconeogenesis, resulting in reduced circulating glucose and insulin concentrations. Insulin-sensitizing effect of metformin could play an important role in reducing the risk of HCC. The preventive effect of metformin on HCC could

also be related to its ability to directly act on tumour cells by promoting growth inhibition, apoptosis and senescence through the activation of 5-adenosine monophosphate-activated protein kinase (AMPK) and the subsequent inhibition of mTORC1 [71, 72]. Studies regarding the potential association between the use of insulin, insulin analogues such as insulin glargine or insulin secretagogues such as sulfonylureas and cancer risk have been controversially discussed [73, 74]. Although no definitive conclusions can be drawn, it seems clear that these drugs have no protective effect on HCC development in patients with diabetes [41, 75, 76].

The concept of selective insulin resistance in the liver

Because insulin resistance is a major feature of CLDs at risk for HCC, linking the activation of insulin pathways through hyperinsulinemia to liver carcinogenesis may seem counterintuitive. One potential hypothesis would be that hepatic insulin resistance is partial and that some pathways remain insulin-sensitive when they are exposed to higher than normal levels of insulin. During CLDs, hepatocytes that have escaped apoptosis and necrosis are exposed to intense selection pressure imposed by inflammation, hyperglycemia, oxidative stress and high circulating levels of free fatty acids, which probably drive cell transformation. The maintenance of some insulin signalling pathways could provide selective advantages for premalignant hepatocytes in terms of metabolism, proliferation and survival.

The concept of selective insulin resistance stems from the fact that, in common insulin-resistant states as observed in type 2 diabetes and obesity, hepatic lipogenesis and lipid production, physiologically activated by insulin, are increased and associated with liver steatosis. Paradoxically, this occurs in concert with increased glucose production, physiologically subjected to insulin-mediated negative

regulation [77, 78]. This concept is highly conceivable because of the complex nature of insulin signalling at the molecular level [17].

Using genetically engineered mice models of insulin resistance, the inability of hyperinsulinemia to suppress hepatic glucose production has been linked to the inability of insulin to inactivate the transcription factor FOXO1 through AKT2-dependent phosphorylation [79, 80]. Consistently, the activity of FOXO1 as well as the mRNA levels of two of its targets, the gluconeogenic genes PEPCK and G6PC, have been reported to increase progressively with the severity of the disease in liver biopsies from patients with NAFLD [81]. Otherwise, IR activation seems to play a crucial role in the establishment of liver steatosis during insulin-resistant states since mice with hepatocyte-specific deletion of IR exhibit marked insulin resistance but lack hepatic steatosis under high-fat diet [82, 83]. Similarly, the hepatic knockdown of IR with chemically-modified antisense oligonucleotides blocks the development of liver steatosis in genetically obese mice [83]. Moreover, humans with inactivating mutations in the IR are extremely insulin-resistant but do not show increased levels of *de novo* lipogenesis and do not develop hepatic steatosis [84]. Downstream of the IR, AKT2 is also required for hepatic lipid accumulation in murine models of obesity and insulin resistance induced by either leptin deficiency or high-fat diet feeding [85]. The failure of insulin to suppress gluconeogenesis while lipogenesis remains activated could be related to the fact that these two pathways are molecularly distinct and display differential sensitivity to the hormone, the lipogenic pathway being less affected by the reduced activation of IR/PI3K/AKT. In this setting, it appears that mTORC1, which is overactivated in the liver from obese rats [86] lies at a bifurcation of insulin signalling pathways upstream of lipogenesis and gluconeogenesis: mTORC1 inhibition blocks insulin-induced upregulation of lipogenic gene expression

but does not affect insulin-mediated suppression of gluconeogenic gene expression [87].

In liver tissue from NAFLD patients, the upregulation of active FOXO1 is correlated to increased expression of IRS-2, increased activation of AKT2 and upregulation of the lipogenic transcription factor SREBP-1c [88, 89]. As FOXO1 has been identified as a positive regulator of IRS-2 expression in murine experimental models [90, 91], it has been proposed that the upregulation of IRS-2 expression and AKT activity by unrestricted FOXO1 activity could represent a permissive mechanism linking hyperinsulinemia with the induction of lipogenesis during NAFLD [89].

PTEN (phosphatase and tensin homolog), a negative regulator of the PI3K/AKT pathway, which dephosphorylates the lipid second messenger phosphatidylinositol 3,4,5-triphosphate (PIP_3), is downregulated in obese patients, insulin-resistant patients and humans with liver steatosis [92]. Liver-specific Pten knockout mice display enhanced steatosis and develop HCC [93, 94]. The downregulation of PTEN could participate to the maintenance of some insulin signalling pathways. Interestingly, insulin has been recently identified as a negative regulator of PTEN expression in *Caenorhabditis elegans* and human breast cancer cells [95]. Whether or not this mechanism occurs in the setting of hyperinsulinemia associated to CLDs remains to be demonstrated. Increased activation of atypical protein kinases C has been also reported in the liver from obese rodents that could explain the effectiveness of hyperinsulinemia to stimulate certain pathways in the liver [96].

Overactivation of insulin-dependent signalling pathways in human HCC

Human HCC tumours have been characterized with respect to insulin signalling and it appears that this pathway is frequently altered and upregulated in a substantial number of cases due to the overexpression of signalling components and the loss of negative regulators. As a consequence, hyperinsulinemia may affect HCC development and progression directly by promoting metabolism, proliferation and survival of cancer cells (Fig. 2).

Quantitative and qualitative changes at the IR level. We have recently reported that IR is significantly overexpressed in 40% of 85 HCC tumours analysed compared to adjacent nontumour tissue [97]. This increase is accompanied by a major modification of the relative expression of the two IR isoforms in ≈70% of HCC samples. The expression of IR-A (-exon 11) is induced while the expression of IR-B (+exon 11) is decreased irrespective of CLD etiology, suggesting that the increase of IR-A/IR-B ratio is a general mechanism of liver oncogenesis. We have demonstrated that the tumoral IR-B to IR-A shift results from the deregulation of IR pre-mRNA alternative splicing that is consecutive to the induction of RNA splicing factors expression from CELF, hnRNP and SF2/ASF families by the epidermal growth factor receptor (EGFR) pathway [97]. IR-A displays approximately 1.8-fold higher affinity for insulin than IR-B and is particularly active in mitogenic signalling in a variety of cancer cells including HCC [97, 98]. It has been reported that IR-A binds also proinsulin with high affinity, which stimulates proliferation in breast cancer cell lines [99]. Proinsulin is present at high levels in the plasma from insulin resistant patients and has been considered until now as a prohormone with little biological activity and no relevance to cancer biology. In view of these new data, proinsulin may play a biological role *via* IR-A activation during cancer development in a context of insulin-resistance. IR-A is also a receptor for insulin-like growth factor-II (IGF-II), a growth

peptide produced by the fetal liver and early after birth, which is re-expressed in some HCC tumours (reviewed in [100]). How these different ligands participate to HCC cell biology *via* IR-A activation deserves further investigation.

Upregulation of IRS expression. The expression of IRS-1 and IRS-2 (at both mRNA and protein levels) is frequently increased in HCC tumours compared to adjacent nontumour liver tissue [101-103]. IRS overexpression has been reported at early stages of liver carcinogenesis in preneoplastic lesions where it may amplify insulin signalling. IRS-1 overexpression is not sufficient to transform murine hepatocytes but it synergizes with the HBx viral protein to promote cellular dysplasia (see below) [104]. In patients with HCC, IRS-1 overexpression correlates with tumour size and tumour progression [101].

Loss of negative regulators of insulin signalling. As mentioned above, CLDs are associated with the up-regulation of SOCS proteins, which act at different levels to inhibit insulin signalling in the liver (reviewed in [24, 37]). Aberrant promoter methylation of SOCS has been frequently identified in HCC in comparison with adjacent nontumour liver tissue [105, 106]. PTEN expression is frequently reduced in human HCC, and is associated with a poor prognosis [107].

Exacerbated lipogenesis. Metabolic changes are key early events during cellular transformation as the synthesis of new membranes and of specific lipids is essential for cancer cell growth and survival. It has been reported that the expression of key lipogenic enzymes and transcription factors is progressively induced from nontumoral liver tissue towards HCC and that aberrant lipogenesis correlates with clinical aggressiveness and poor survival [108-110]. The exacerbation of lipogenesis is essential for insulin signalling in HCC cells. Indeed, the abrogation of lipid synthesis through the silencing of lipogenic enzymes (fatty acid synthase, acetyl-coenzyme A

carboxylase, stearoyl-CoA desaturase 1) or lipogenic transcription factors (SREBP-1c) with RNA interference reduces insulin-dependent proliferation and survival in HCC cell lines [109, 111].

Warburg effect. A metabolic remodeling, shifting mitochondrial respiration to predominant cytosolic glycolysis called Warburg effect has been involved in the early stages of hepatocyte transformation. Upregulation of the glycolytic enzymes pyruvate kinase M2 (PKM2), hexokinase 2 and lactate deshydrogenase and inhibition of mitochondrial biogenesis and functions, increase insulin-activated glucose metabolism, providing a metabolic advantage for HCC cell growth [112, 113].

Indirect effets of hyperinsulinemia

Hyperinsulinemia may affect liver cancer development and progression not only through direct effets on the growth of preneoplastic and transformed hepatocytes but also indirectly by increasing the production of cytokines and mitogens, enhancing fibrosis and promoting angiogenesis (**Figure 2**).

IGF-I/IGFBP pathway. IGF-I is a mitogen that is primarily produced in the liver and shares sequence homology with insulin. IGF-I has a role in cancer development and high serum IGF-1 levels are associated with an increased risk of cancers such as colon and breast cancers (reviewed in [114]). Chronic hyperinsulinemia may lead to increase circulating levels of free and bioactive IGF-I because i) insulin stimulates growth hormone receptor expression which controls IGF-I production and secretion in the liver and ii) insulin decreases the hepatic synthesis and blood levels of IGF-binding proteins (IGFBP)-1 and -2 (reviewed in [115]). In the setting of CLDs, it is plausible that chronic elevation of insulinemia increases levels of IGF-I at early but not advanced stage of diseases. Indeed, IGF-I serum levels have been found to

decrease gradually when compared between healthy subjects, patients with cirrhosis and patients with HCC (reviewed in [116]). A significant inverse association has been found between IGF-I and insulin levels in patients with HCC [117]. IGF-I deficiency in HCC is thought to result from the reduced synthesis capacity of the cirrhotic liver mass. In addition, some cytokines, including IL-1β, TNF-α and IL-6, and miRNA such as miR-190b, which are elevated in patients with HCC have been reported to block IGF-I production in the liver [117].

Leptin. Leptin, the product of the (*ob*) gene, is a pleiotropic adipokine best known as a regulator of food intake and energy expenditure *via* hypothalamic-mediated effects. Plasma leptin levels are abnormally high in obese patients and exhibit a positive correlation with the amount of fat mass and the degree of insulin resistance. Insulin regulates leptin availability by stimulating both leptin synthesis and the release of leptin from pre-existing intracellular pools in adipocytes and cancer cells [118, 119]. The role of leptin during CLDs and liver carcinogenesis is complex (reviewed in [120, 121]). Leptin plays profibrogenic and proangiogenic roles by acting on hepatic stellate cells and Kupffer cells to synthetize extracellular matrix components, proinflammatory or proangiogenic cytokines [122-124]). Leptin promotes HCC cell proliferation, migration and invasion [125, 126]. These data suggest that hyperinsulinemia could favour HCC development and progression through leptin-dependent mechanisms. However, leptin has also been shown to inhibit ectopic lipid storage, therefore limiting cellular lipotoxicity, in particular in the liver [127]. Consistently, leptin deficiency secondary to obesity with mutations in the *ob* gene, or to lipodystrophic syndromes due to genetically-determined reduced fat amount, is associated with severe insulin resistance and liver steatosis, which are reversed by leptin substitutive therapy [128].

Fibrosis. There is substantial evidence linking insulin resistance and fibrosis in patients with CLD. In HCV patients, the degree of insulin resistance parallels the liver fibrosis stage and insulin resistance is an independent predictor of fibrosis degree and progression [129-131]. Insulin resistance is also an independent predictor of advanced fibrosis in NAFLD [130]. The measurement of the liver stiffness, an accurate noninvasive diagnosis marker of liver fibrosis, is significantly correlated with HOMA-IR in NAFLD [132]. In a study of 782 patients with NAFLD, HOMA-IR was positively associated with advanced fibrosis (> stage 2) [133]. The link between insulin resistance and fibrosis could result from the ability of insulin to directly activate hepatic stellate cells which, as shown *in vitro,* leads to increased expression of key fibrotic proteins such as connective tissue growth factor and extracellular matrix production [134, 135]. As mentioned above, the ability of insulin to stimulate leptin expression could also play a contributing role in the hormonal promotion of liver fibrosis.

Angiogenesis. HCC is a highly vascularized tumour, relying on the formation of new blood vessels for growth. Vascular endothelial growth factor (VEGF) is critical in this process and anti-angiogenic therapies such as sorafenib possess a therapeutic potential in advanced HCC [136]. Insulin has been reported to directly stimulate vascular endothelial cell proliferation, migration and capillary tube formation *in vitro* and angiogenesis in animal models (reviewed in [137-139]). Insulin is also a potent upregulator of VEGF mRNA expression and VEGF release from adipocytes, vascular endothelial cells and cancer cell types, thus regulating neovascularization indirectly [140, 141]. Insulin promotes sinusoidal endothelial cell proliferation through VEGF upregulation in regenerating rat liver after partial hepatectomy [142]. As mentioned above, insulin induction of leptin expression in adipocytes could also be an endocrine

mechanism whereby insulin could promote angiogenesis. As angiogenesis is also essential to the metastatic dissemination of tumour cells to distant organs, insulin is expected to play a prominent role in this process. Accordingly, IR downregulation in breast cancer cell lines with short hairpin RNA reduces VEGF expression and cell metastatic potential after graft into the mammary fat pad of athymic mice [143]. Similarly, mammary tumour metastasis is significantly diminished in the absence of IRS (reviewed in [144]).

Advances from animal models

Because of the complex interaction between metabolic dysregulations and HCC, animal models have been helpful to dissect and increase the understanding of molecular links between hyperinsulinemia and HCC.

Intrahepatic transplantation of pancreatic islets in rats. Dombrowski and colleagues have developed two animal models of pancreatic islet transplantation into the liver of streptozotocin-induced diabetic rats and of genetically diabetic BB/Pfd rats [145-147]. In these models, as a low number of islets is transplanted, glycemia is not efficiently controled and a mild systemic hyperglycemia persists which constitutes a constant stimulus for the islet grafts to synthesize and secrete insulin. Strikingly, in the first three months after transplantation, morphological changes are observed in the liver acini located downstream of the islet graft, which are reminiscent of preneoplastic foci observed in chemically-induced liver carcinogenesis: excessive storage of glycogen and lipids combined with a high hepatocyte turnover. These lesions gradually expand into the liver and progress to HCC within 6 to 24 months. At the molecular level, the IR/IRS-1/AKT/mTOR pathway is markedly dysregulated together with fatty acid synthesis and glycolysis in the hepatocellular lesions [111, 147]. These

models highlight that local hyperinsulinism in the rat liver triggers a number of metabolic alterations that are associated with preneoplastic foci and HCC occurrence.

IRS1-kockout mice. C57BL/6J mice submitted to long-term high fat diet become obese, insulin resistant, and successively develop steatosis, NASH and liver tumours. The knockdown of IRS-1 in this genetic background dramatically protects mice against high fat diet-induced NASH and liver tumourigenesis despite severe insulin resistance and hyperinsulinemia [148]. These findings suggest that the maintenance of hepatic insulin signalling pathways through IRS-1 is required for the development of NASH and HCC in the setting of insulin resistance and support the concept of selective insulin resistance during CLD.

IRS-1 overexpressing mice. Transgenic mice that constitutively overexpress IRS-1 in the liver never develop HCC indicating that overexpression of IRS-1 alone is not sufficient to cause hepatocellular transformation [149]. In contrast, combined overexpression of IRS-1 together with HBx viral protein has a synergistic effect to promote dysplasia and HCC through both IR/IRS-1/MAPK and Wnt/β-catenin cascades [104].

AB6F1 mice. By intercrossing A/JCr with C57BL/6J mice, Hines and colleagues have identified male F1 offspring that develops spontaneous hyperinsulinemia, severe NAFLD by 9 months and HCC by 15 months on a standard chow diet in the absence of obesity or type 2 diabetes [150]. Hyperinsulinemia is associated with an adipogenic transition of hepatocytes involving the induction of expression of PKM2, an anabolic enzyme implicated in tumorigenesis and the Warburg effect [151]. This model of primary insulin resistance supports the hypothesis that hyperinsulinemia is a critical feature to promote the progression from NAFLD to HCC in mice.

Therapeutic targeting of IR signalling

Several therapeutic strategies can be considered to reduce IR-dependent signalling in the course of CLD and HCC. As insulin has emerged as a new candidate biomarker to refine HCC risk assessment beyond established risk factors, medications that improve insulin resistance (such as metformin) deserve further investigation for HCC prevention.

IGF-II, a ligand for IR-A, is a potential therapeutic target in HCC. Thus the growth of human HCC cell lines is blocked *in vitro* and *in vivo* with DX-2647, a human monoclonal antibody against IGF-II [152]. BI 836845, a monoclonal antibody to IGF-II and MEDI-573, a dual-targeting monoclonal antibody to IGF-II/IGF-I are currently under clinical investigation for solid tumours including HCC (https://clinicaltrials.gov). This anti-ligand approach is of particular interest since it will result in inhibition of IR-A signalling without interfering with IR-B signalling.

To date, there is no available antibody blocking selectively IR-A. Several antibodies against the structurally related IGF-1R have been developed. These antibodies are highly specific to IGF-1R and spare IR. Unfortunately, the use of these antibodies is associated with a "metabolic toxicity", resulting in increased levels of growth hormone, IGF-I, insulin and glucose [114]; supraphysiological levels of IGF-I and insulin may lead to the adverse stimulation of IR. In addition, we have reported that the anti-IGF-1R antibody AVE1642 induced a compensatory resistance mechanism involving the EGFR/HER3 pathway In HCC cell lines [153]. A phase II study evaluating the anti-IGF-1R cixutumumab (IMC-A12) in unselected patients with advanced HCC did not show meaningful activity [154].

Due to the high homology between the catalytic domains of IGF-1R and IR, dual IGF-1R/IR tyrosine kinase inhibitors have been developed for clinical oncology. While hyperinsulinemia is likely in treated patients, these drugs have the potential to suppress any deleterious effects of insulin in cancer cells. A pre-clinical study shows that the IGF-1R/IR inhibitor OSI-906 is efficient in human HCC cell lines expressing high levels of IGF-II and IR [155]. Two phase II clinical trials testing a continuous dosing regimen of OSI-906 in patients with advanced HCC after failure of sorafenib or in combination with sorafenib have been stopped probably due to safety issues (https://clinicaltrials.gov). It seems that intermittent dosing regimen of OSI-906 should reduce toxicity [156].

Concluding remarks

- Insulin resistance and compensatory hyperinsulinemia are strikingly linked to CLDs, whatever their underlying cause. Clinical and epidemiological data together with studies in both humans and animal models of insulin resistance now suggest that high circulating concentrations of insulin *per se* are significantly associated with an increased risk of HCC, independently of established liver cancer risk factors and obesity parameters.

- These data have important implications for the clinical management of patients with CLD. Efforts to manage hyperinsulinemia should be considered in patients with any form of CLD. Due the increased burden of diabetes and obesity worldwide, these conditions would plausibly account for more NASH-related HCC cases in the future. Effective treatments of insulin resistance and hyperinsulinemia are likely to enhance liver cancer prevention and improve disease outcomes.

- It is highly probable that hyperinsulinemia collaborates with other environmental factors to induce multiple hits that are required for liver tumorigenesis and progression. Further research is needed to explore the relative importance of hyperinsulinemia in these processes. Elucidating the signalling pathways for HCC development in the setting of hyperinsulinemia will increase our understanding of the pathogenesis of HCC and might promote the development of novel preventive and therapeutic strategies.

Acknowledgements

Legends to Figures

Figure 1: Insulin signalling in hepatocytes.

Upon insulin binding, a conformational change and autophosphorylation of the insulin receptor (IR) occur leading to the recruitment and phosphorylation of IR substrates such as IRS-1, IRS-2 and Shc proteins and to the initiation of cascades of phosphorylation events. IRS proteins mostly activate the PI3K-Akt pathway by recruiting and activating PI3K, leading to the generation of second messenger PIP_3. Membrane-bound PIP_3 recruits and activates PDK-1, which phosphorylates and activates Akt. Akt mediates most of insulin's metabolic effects, regulating glycogen, protein and lipid syntheses as well as gluconeogenesis. Akt also plays a role in the control of cell cycle and survival. The Shc-Grb2-Sos-Ras pathway rather controls cellular proliferation. For glucose metabolism, insulin increases the expression of glucokinase (GCK), the enzyme responsible for the first step of glycolysis. It suppresses the expression of the cytosolic form of phosphoenolpyruvate carboxykinase (PEPCK) and glucose 6-phosphatase catalytic subunit (G6PC), the first and last steps of gluconeogenesis, respectively. For lipid metabolism, insulin increases the expression level of sterol regulatory element-binding protein 1c (SREBP-1c), a transcription factor critical for fatty acid biosynthesis through the upregulation of the lipogenic enzymes acetyl-CoA carboxylase (ACC), fatty acid synthase (FASN) and stearoyl-CoA desaturase 1 (SCD1).

Figure 2: Pathogenic pathways that may link hyperinsulinemia to HCC development and progression.

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diabetes   obesity   metabolic
                        syndrome

insulin resistance

NAFLD
↘insulin degradation

chronic inflammation
oxidative stress
lipotoxicity

hyperinsulinemia

virus   alcohol

chronic liver disease

insulin resistance
↘insulin degradation

hepatic stellate cell

hepatocyte transformation

IR-B >>IR-A   IR-A >>IR-B

endothelial cell

fibrogenesis
inflammatory cytokines
angiogenic cytokines

metabolism
proliferation
survival
invasion

angiogenesis