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Non-alcoholic fatty liver disease and diabetes mellitus as growing aetiologies of hepatocellular carcinoma

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Running Title: Molecular insights of NAFLD and diabetes as risk factors for HCC

Summary

Obesity-related complications such as non-alcoholic fatty liver disease (NAFLD) and type 2 diabetes (T2D) are well-established risk factors for the development of hepatocellular carcinoma (HCC). This review provides insights into the different molecular mechanisms impacted by fat accumulation, hyperinsulinemia and inflammation in the liver, leading to HCC progression. We focus on recent findings linking intracellular pathways and transcription factors that can trigger the reprogramming of hepatic cells. In addition, we highlight the role of enzymes in dysregulated metabolic activity and consequent dysfunctional signalling. Finally, we discuss the potential uses and challenges of novel therapeutic strategies to prevent and treat NAFLD/T2D-associated HCC.

Keywords: Hepatocellular carcinoma, obesity, non-alcoholic fatty liver disease, type 2 diabetes, hepatocyte transformation

Abbreviations: *ACACA: acetyl-CoA carboxylase alpha, ACLY: ATP citrate synthase, ATP-citrate lyase, AKT: protein kinase B, AMPK: AMP-activated protein kinase, AP: activator protein-1, BCL-2: B-cell lymphoma 2, BIM: Bcl-2 Interacting Mediator of cell death, BMP4: bone morphogenetic protein 4, CD: cluster of differentiation, CRP: C-reactive protein, CTNNB1: Catenin Beta 1, DAG: diacylglycerol, DEN: diethylnitrosamine, ECM: cell-extracellular matrix, ELF3: E74 Like ETS Transcription Factor 3, ER: endoplasmic reticulum, ERα: estrogen receptor α, FASN: fatty acid synthase, FGF2: fibroblast growth factor 2, FRA-1: fos-related antigen 1, GCKR: glucokinase regulator, GLP-1RA: GLP-1 receptor agonists, GSR: glutathione reductase, GWAS: genome-wide association study, HCC: hepatocellular carcinoma, HFD: high-fat diet, HGF: hepatocyte growth factor, HLA: human leukocyte antigen, HLCs: hepatocyte-like cells, HSD17B13: 17β-hydroxysteroid dehydrogenase type 13,*

IGF: insulin-like growth factor, IKK-β: inhibitor of nuclear factor kappa-B kinase subunit beta, IL: interleukin, iPSCs: induced pluripotent stem cells, IR: insulin receptor, JAK: janus kinase, LAMs: lipid-associated macrophages, JNK: c-Jun N-terminal kinases, LXR: liver X receptor, mTOR: mammalian target of rapamycin, MBOAT7: membrane-bound O-acyltransferase domain containing 7, moKCs: monocyte-derived KCs, *MUFA: monounsaturated fatty acid, NAFLD: nonalchocolic fatty liver disease, NASH: nonalcoholic steatohepatitis, NF-κB: Nuclear factor kappa-light-chain-enhancer of activated B cells, NRF-2: nuclear factor erythroid 2–related factor 2, OSM: Oncostatin M, PC: pyruvate carboxylase, PI3K: phosphatidylinositol 3-kinase, PNPLA3: patatin-like phospholipase domain-containing 3, PPAR: peroxisome-proliferator activated receptor, PTP: protein tyrosine phosphatases, PTEN: phosphatase and tensin homolog, PUFA: polyunsaturated fatty acid, resKC: Hepatic resident Kupffer cells, ROS: reactive oxygen species, SFA: saturated fatty acid, SGLT2: sodium-glucose cotransporter-2, SNP: single-nucleotide polymorphism, SOX9: SRY-Box Transcription Factor 9, SREBP-1, -2: sterol regulatory element binding protein-1, -2, STAT: signal transducer and activator of transcription, TCA: tricarboxylic cycle, TGF: transforming growth factor, TM6SF2: transmembrane-6 superfamily member-2, TNF: tumour necrosis factor, TLR: tol-like receptor, TrxR1: thioredoxin reductase-1, TZDs: thiazolidinediones, T1D: type 1 diabetes, T2D: type 2 diabetes, SHP: Src homology region 2 domain-containing phosphatase, SNP: Single nucleotide polymorphisms, VEGF: vascular endothelial growth factor, ZBTB20: zinc finger and BTB domain containing 20.*

Key Points

- NAFLD and T2D are among the fastest growing aetiologies in HCC development.
- Hyperinsulinemia, dysregulated glucose homeostasis and increased lipid accumulation can activate pathways that promote hepatic tumour development in NAFLD and T2D onset.
- Hepatic inflammation, oxidative stress and insulin resistance are important hallmarks of NAFLD/T2D-related HCC.
- Antidiabetic drugs, like metformin and TZDs, reduce HCC risk, yet their therapeutic effect can be contradictory in advanced stages.
- The stratification of HCC patients in clinical trials should consider the presence of diabetes, due to its impact on incidence and prognosis.

Introduction

Hepatocellular carcinoma is an aggressive and treatment-resistant cancer and represents the third most common cause of cancer-related death [1]. Primary liver cancer includes hepatocellular carcinoma (HCC, comprising 75%-85% of cases) intrahepatic cholangiocarcinoma (comprising 10%-15% of cases), and other rare types. The highest HCC incidence and mortality are observed in Asia and Africa but are also increasing worldwide, especially in Europe and the U.S.A. [2].

Non-alcoholic fatty liver disease (NAFLD) is a risk factor that contributes to HCC development. NAFLD can progress to non-alcoholic steatohepatitis (NASH) in 20-30% of cases, and approximately 20-25% of NASH patients progress to cirrhosis [3], which is the strongest risk factor for HCC development. NAFLD is the leading cause of chronic liver disease worldwide [4, 5]. There is an unmet need to accurately identify metabolic risk factors that can better predict advanced stages of the disease and related complications [6].

Diabetes is a metabolic disorder characterised by impaired regulation of glucose and insulin levels. The prevalence is exceptionally high, with an estimated 463 million diabetic patients in 2019, accounting for 9.3% of the adult human population [7]. There are three major forms: autoimmune type 1 diabetes (T1D), insulin-resistance-associated type 2 diabetes (T2D), and monogenic forms of diabetes. However, this classification is currently under re-evaluation [8]. T2D is considered a metabolic risk factor for the development of NAFLD, advanced fibrosis, and HCC [4, 6]. Simon et al. demonstrated in two well-characterized population cohorts that T2D is an independent risk factor for HCC development [6]. T2D is significantly associated with severe liver disease [9], furthermore patients with advanced NAFLD (NASH with severe fibrosis), have a higher incidence of T2D [4, 10]. It is unclear whether NAFLD drives T2D, or if hyperglycaemia/hyperinsulinemia pushes NAFLD towards an advanced stage,

indicating that most likely the pathological processes are intertwined. Therefore, the underlying mechanisms by which NAFLD/T2D can promote HCC development are not completely understood.

In this review, we discuss the pathogenic pathways activated by nutrient overload and the intracellular mechanisms that lead to aberrant signalling and hepatocyte reprogramming. We also review the involvement of inflammation in the transition from NAFLD/T2D to HCC. Finally, we will discuss current and new therapeutics for treating HCC and emerging technologies that will accelerate the translational process.

Risk factors involved in HCC progression in NAFLD and T2D

Genetics

To date, there are no genome-wide association studies (GWAS) that have defined the genetic variations associated with NAFLD-HCC risk, in either presence or absence of cirrhosis. However, single-nucleotide polymorphisms (SNPs) in genes that promote fat accumulation in hepatocytes have been identified as genetic risk factors in NAFLD, T2D, and HCC [11-13]. For example, the rs738409 polymorphism in *PNPLA3* (phospholipase domain-containing 3) and the rs58542926 polymorphism in *TM6SF2* (transmembrane 6 superfamily member 2) have been strongly associated with early steatosis and more advanced NAFLD and NASH [14]. *PNPLA3* rs738409 was also found at a higher frequency in a cohort of HCC patients with T2D [15]. Furthermore, the *TM6SF2* rs58542926 polymorphism was associated with fatty liver and higher T2D risk in a GWAS of >300,000 participants [16]. However, in other studies, the *PNPLA3* polymorphism was linked to an increase in liver fat, but it was not found to be associated with insulin resistance [17].

More recently, a polygenic risk score has been developed to predict HCC in patients with obesity-related metabolic disorders and to improve HCC risk stratification [18]. This polygenic risk score combines SNPs in *PNPLA3* and *TM6SF2*, with other SNPs in membrane bound O-acyltransferase domain containing 7 (*MBOAT7*), glucokinase regulator (*GCKR*) and 17β-hydroxysteroid dehydrogenase type 13 (*HSD17B13*) [18].

NCOA5, also known as coactivator independent of AF2 (CIA), is a coregulator of estrogen receptor (ERα)-mediated transcription. Reduced NCO5 expression has been associated with patients with T2D and HCC [19]. Remarkably, heterozygous deletion of the *Ncoa5* gene in mice led to HCC through its effects on hepatic IL-6 expression [19, 20].

Carbohydrates, diabetes development and progression to HCC

An excessive intake of simple carbohydrates is associated with obesity and metabolic syndrome [21]. It was estimated that a 20% reduction in added sugars intake by 2035 will reduce obesity, T2D, coronary heart disease as well as liver complications such as hepatic steatosis, NASH, cirrhosis and HCC in the U.S.A. [22].

Fructose is a simple sugar whose intake has dramatically increased in the western diet through sweetened beverages. Wali *et al.* showed that mice fed with a 50:50 mixture of fructose and glucose diet had strong induction of the lipogenesis pathway [23]. Similarly, in healthy individuals, co-ingestion of fructose and glucose led to an increase in lipogenesis [23]. In addition, improved cardiometabolic health was observed when mice were fed diets containing resistant starch, instead of native starch and low protein content [23]. These findings indicated that the type of carbohydrate and protein availability in the diet can provide metabolic benefits (**Fig. 1**).

Softic *et al.* demonstrated that mice fed with a HFD supplemented with fructose developed a more severe metabolic phenotype, compared to mice on a HFD supplemented with glucose [24]. Fructose supplementation led to obesity, glucose intolerance and impaired insulin signalling. SREBP1c, a master lipogenic regulation, gene expression and downstream lipogenesis genes were also activated, resulting in deteriorated insulin signalling [24]. Fructose is metabolized by fructokinase (ketohexokinase), the first enzyme in fructose metabolism. In hepatocytes, fructokinase stimulation induces lipogenesis and fat accumulation [25]. Mice on a high-fructose diet had increased lipogenesis together with NASH and HCC development [25]. Accordingly, loss of fructose metabolism is observed in HCC patient samples, and ketohexokinase overexpression in liver cancer cells leads to decreased fructose flux through glycolysis [26].

Ketogenic diet, protein intake and liver dysfunction

A ketogenic diet limits carbohydrate intake, which results in low glucose levels, thus reducing lipogenesis [27]. Ketogenesis leads to ketone body production, which represents an energy source in a state of nutrient deprivation, such as prolonged fasting and starvation. Clinical trials have shown the benefits of the ketogenic diet for weight loss; however, its use remains controversial due to reports showing a worsened metabolic outcome [28]. Caloric restriction can slow down the ageing process by activating reprogramming of liver metabolism. Mice subjected to high energy intake, or high caloric intake, showed an increase in proteins involved in nutrient metabolism, including glycolysis, gluconeogenesis, tricarboxylic acid cycle, lipogenesis, β-oxidation, amino acid metabolism and ketogenesis [29]. Low energy intake instead was associated with RNA metabolism and splicing upregulation. Metformin, rapamycin and

resveratrol, known for prolonging lifespan in animal models, play a role in reverting changes obtained with the high energy and macronutrient intake. Through mTOR inhibition, these agents lead to a reduction in proteins and downstream splicing pathways [29]. Interestingly, mice fed with a low-protein content diet showed improved metabolic health with increased mitochondrial activity [30]. Conversely, excessive protein intake increases mitochondrial function and is also associated with oxidative stress [29], which accelerates the ageing process and contributes to HCC development [31]. The "right" balance between protein, carbohydrate and fat intake in health and disease is still controversial. Evaluation in the obese population and in T2D patients under treatment will help to clarify the real incidence in HCC.

Insulin resistance and lipid metabolism

The liver is a key regulator of glucose and lipid metabolism, excessive hepatic lipid accumulation and increased glucose production characterize NAFLD and T2D. [32]. In several epidemiological studies, both NAFLD and T2D have been identified as significant risk factors for the development of HCC [33, 34]. Hepatic insulin resistance significantly contributes to T2D through fat-induced dysfunctional signalling of the insulin receptor (IR). Dysregulated IR signalling leads to aberrant downstream activation of PI3K/AKT, which prevents insulin from inhibiting gluconeogenesis in the liver (**Fig. 1**). Insulin resistance induces elevated circulating insulin levels, which stimulates increased insulin-like growth factor-1 (IGF-1) production and subsequently upregulates proliferation and prevents apoptosis in hepatocytes, contributing to HCC [35]. Hyperinsulinemia also activates insulin receptor substrate-1 (IRS-1) which is associated to HCC development [36].

Daily lipid overload with inadequate mitochondria function contributes to the increased production of diacylglycerols (DAG) and ceramides, which promote insulin resistance, NAFLD and eventual HCC development [17, 37, 38]. The degradation of ceramides is associated with improved insulin sensitivity and decreased inflammation [39]. Several studies have found that DAG accumulation can lead to hepatic insulin resistance via activation of protein kinase C (PKC). Phosphorylation of the insulin receptor by PKC was found to impair insulin signalling [40]. Additionally, increased hepatic DAG content in humans was linked to hepatic insulin resistance, which was also associated to PKC activation [41]. This DAG-PKC axis was found to be the strongest predictor of insulin resistance in obese patients. Dysfunctional insulin signalling can, in turn, increase lipid accumulation by a mechanism known as selective insulin resistance [42]. Dysfunctional insulin signalling contributes to *de novo* lipogenesis (DNL) and increased hepatic fat accumulation promotes insulin resistance, leading to a vicious cycle linked to the progression of T2D and advanced NAFLD.

Liver steatosis occurs when fatty acid uptake and DNL are elevated over fatty acid oxidation and secretion. DNL was found to be positively associated with hepatic saturated fatty acid (SFA) content; both DNL and SFA levels are elevated in NAFLD and T2D patients [43]. In addition, SFA content was negatively correlated with hepatic insulin sensitivity [43] and dysregulation of lipid metabolism correlates with the progression of liver disease to HCC [44]. Thus, lipid metabolism can be drastically reprogrammed in malignant hepatic cells.

Several lipogenic enzymes, such as ACLY and ACACA, are upregulated in liver cancer [45]. Specifically, fatty acid synthase (FASN), a key enzyme in lipogenesis, is upregulated in HCC patients and may be an important driver in cancer development

[46, 47]. Indeed, in an HCC mouse model, deletion of *Fasn* prevented hepatocarcinogenesis in mice with oncogenic overexpression of c-Met/AKT and AKT alone [47]. Additionally, FASN inhibition was found to suppress HCC formation in c-Myc overexpressing tumours [48]. However, FASN was also found to be dispensable in a murine HCC model with c-Met and β-catenin overexpression [49]. These studies highlight that the role of FASN and DNL in hepatocarcinogenesis is oncogene dependent, which has important implications in designing targeted treatment options.

Peroxisome proliferator-activated receptor (PPAR)-γ is a nuclear receptor protein that is a key regulator in lipid metabolism. In the liver, PPAR-γ is an early contributor to NAFLD development (**Fig. 1**), where it increases steatosis by upregulating DNL and FFA uptake [50]. PPAR-γ was found to be elevated in the livers of obese patients with NAFLD [51, 52]. Paradoxically, PPAR-γ has been also found to suppress tumorigenesis, inhibiting PI3K/AKT-mediated apoptosis and cell arrest in HCC [53]. Thus, PPAR-γ agonists have been investigated in clinical trials as potential therapeutic agents for HCC [54, 55], but their use remains controversial given metabolic adverse effects.

Obesity and T2D, have also been linked to lipogenic regulator SREBP1 expression [56]. mTORC1 and mTORC2 increase SREBP1 transcription and are major upstream contributors to lipogenesis regulation. mTORC1 is responsible for lipid synthesis by SREBP1 activation during insulin resistance, contributing to hepatic steatosis [56]. SREBP1 is elevated in liver tumour tissue [57] and its inhibition has been also proposed as a therapeutic strategy for HCC [58, 59].

FFA synthesis is increased in tumoral cells for membrane support and energy production, promoting cancer growth and metastasis formation [45]. In a study investigating the lipidomic profile of NAFLD-associated HCC patients, a decrease in

unsaturated fatty acids and acylcarnitines was found in blood circulation, as well as an increase in fatty acid transporters in tumours [60]. HCC patients had decreased levels of free carnitines and increased levels of long-chain acylcarnitines. Notably, low serum levels of acetylcarnitine were identified as a strong candidate biomarker of HCC development [61]. In a proteomic and lipidomic study of mice and humans, lipid-modifying enzymes were found to convert SFAs to MUFAs in HCC, and an increased ratio of long-chain n6-PUFAs over n3-PUFAs in NASH is associated with higher HCC risk [62].

Overall, these findings highlight the important role of altered insulin resistance and lipid metabolism in liver disease and may be crucial drivers in the progression from NAFLD and T2D to HCC and early disease diagnosis.

Hepatic inflammation, a key component of NAFLD/T2D-related HCC development

Inflammatory pathways

The liver is well known for its role in metabolism and detoxification, yet it also has an essential role in the body's immune response. Almost all subsets of leukocytes and phagocytes can be present in the liver, while the largest population of hepatic immune cells are Kupffer cells, liver resident macrophages [63]. Hepatic cells must maintain a balance of tolerance to immune cells during normal physiological function, while also remaining protected against foreign pathogens and tissue damage. Consequently, the hepatic immune cell population can be significantly altered in NAFLD, T2D and HCC [64] (**Fig. 2**).

NAFLD and T2D are characterized by chronic low-grade inflammation, this sustained immune-mediated damage has been linked to HCC development (**Fig. 2**). In HCC patients, pro-inflammatory cytokines such as IL-6, TNF-α, and C-reactive protein (CRP) levels are elevated, suggesting enhanced inflammation and insulin resistance [65, 66]. Chemokines have also been linked to NAFLD and HCC progression. NAFLD-associated HCC patients compared to NAFLD patients without HCC were found to have higher plasma levels of IL-8, IL-13, CCL-3, CCL-4, and CCL-5, which was correlated with activated circulating monocytes [67].

Cytokines act as a balance between immune tolerance and inflammation in the liver microenvironment. In hepatocytes, IL-6 and TNF-α can activate several signalling pathways linked to inflammation, steatosis and oncogenesis. In a mouse model of obesity, elevated expression of IL-6 and TNF-α promoted liver fat accumulation and inflammation [68]. Furthermore, this inflammatory response and hepatosteatosis induced oncogenic STAT3 activation and promoted HCC development [68].

NF-κB

NF-κB signalling is linked to increased insulin resistance in obesity and T2D models, where it is induced by low grade inflammation [69, 70]. HCC patients were found to have elevated NF-kB activity [70]. When NF-kB-activating kinase IKKβ was constitutively activated in hepatocytes, mice exhibited hyperglycaemia as well as hepatic and system insulin resistance [69]. An increase in pro-inflammatory cytokines and inflammatory signalling was also found in IKKβ-activated mice. Thus, NF-κB activation in hepatocytes can lead to a diabetic phenotype. Interestingly, the hepatocytic IKK:NF-κB axis also regulates lipogenesis and cholesterol synthesis, independent of its central role in inflammation [71].

Induction of NF-κB by obesity-associated inflammation can also lead to insulin resistance via a phosphotyrosine signalling mediated mechanism. Activated NF-κB was found to bind and overexpress hepatic tyrosine phosphatase PTPR-γ in obesity/T2D mouse models [72]. This elevated PTPR-γ activity was linked to significant inflammation and insulin resistance in mice and humans. Upon PTPR-γ loss in mouse models, glucose production was decreased and hepatic insulin signalling was enhanced [72]. Thus, NF-κB/PTPR-γ balance affects hepatic metabolism, which is dysregulated by obesity-associated inflammation and can contribute to HCC.

NF-κB has pro-tumorigenic properties where its activation promotes HCC cell proliferation, survival, and invasion. NF-κB can also activate stromal and immune cells, enhancing inflammation and fibrosis [70]. Paradoxically, loss of NF-κB has also been found to significantly promote HCC development [70, 73, 74]. NF-kB-activating kinase IKKβ can prevent liver tumorigenesis by suppressing hepatocyte cell death and proliferation. In a late-stage HCC mouse model, IKKβ-knockout mice showed a significant increase in tumour number and size [75]. IKKβ was identified as a negative regulator of HCC development through ROS-mediated STAT3 signalling [75]. It is not uncommon for both the hyperactivation and inactivation of pathways to result in similar outcomes in biology, albeit through different mechanisms. As a key mediator in inflammation and survival, understanding the context-dependent role of NF-κB in liver disease requires further investigation.

JAK-STAT

The JAK-STAT pathway is a key regulator in inflammation, insulin resistance, T2D, and HCC. The members of this pathway are signal transducer and activator of transcription (STAT) and Janus kinase (JAK). Cytokines and growth factors activate

JAK-STAT signalling which leads to the expression of downstream gene targets involved in cell proliferation, survival, stress and immune responses [76].

As previously described, STAT3 has been found to be constitutively activated in HCC tumours and induced by pro-inflammatory IL-6 [77, 78]. This transcription factor is involved in tumour initiation and promotion, furthermore phosphorylated STAT3 was found in 60% of human HCC patients and associated with more aggressive tumours [79].

JNK

In obesity, the JNK family acts as a critical regulator in insulin resistance and NASH. Elevated c-Jun-JNK activity has been identified in the livers of obese patients, which was subsequently linked to hepatic insulin resistance and steatosis. JNK1 and JNK2 were found to negatively regulate insulin sensitivity and glucose uptake in HFD-fed mice [80]. We found that hepatic fat accumulation activated JNK signalling, which lead to an increase in the expression of the BCL-2 member BIM [81]. In a liver-specific BIM knockout mouse model, insulin sensitivity was improved while hepatic steatosis was reduced [81]. BCL-2 proteins are important modulators of cell survival and are often dysregulated in cancer, including HCC [82].

In addition to its role in liver steatosis, JNK signalling can promote tumour initiation in HCC. JNK-activation of oncogenic c-Myc led to the downregulation of tumour suppressor p21 in hepatocytes [83]. However, the pro-tumorigenic role of JNK seems to be associated to nonparenchymal cells causing expression of pro-inflammatory cytokines IL-6 and TNF-α. [84]. This association between activated JNK signalling, inflammation and HCC development has been identified as an attractive target for therapy.

Kupffer cells

Hepatic resident Kupffer cells (resKCs) are considered as one of the pro-inflammatory drivers in the development of T2D/NAFLD-related HCC. However, it was recently revealed that resKCs were depleted in NAFLD and were instead replaced by two subsets of pro-inflammatory recruited and activated macrophages: monocyte-derived KCs (moKCs) and hepatic lipid-associated macrophages (LAMs) [64]. The latter subset was activated in obesity and able to metabolize lipids (**Fig. 2**). LAMs were also found to be frequently accumulated in liver regions with increased pro-fibrotic Desmin, produced by hepatic stellate cells [64]. Interestingly, another study demonstrated that when the insulin signalling pathway was inhibited, macrophages showed an anti-inflammatory phenotype and had lower expression of IL-6, IL-1β, and TNF-α [85]. This altered macrophage heterogeneity highlights that Kupffer cell lineage and activation is important in NAFLD and HCC. Subsets of recruited and activated macrophages may be responsible for increased inflammation and fibrosis in NAFLD progression to HCC.

T cells

Adaptive immunity has also been shown to play a role in HCC development. In diet-induced mouse models, there was a liver-specific loss of $CD4^+$ T cells but not $CD8^+$ T cells [86]. Excessive lipid accumulation in hepatocytes led to linoleic acid secretion and induced ROS-mediated $CD4^+$ T cell death. This hepatic depletion of $CD4^+$ lymphocytes was strongly associated with increased tumorigenesis [86]. However, IR knockout in T cells had reduced production of pro-inflammatory cytokines upon activation and diminished cytotoxicity [87].

The role of T cells has also been explored in NASH, where a specific subset of auto-aggressive CXCR6$^+$ CD8 T cells were identified in preclinical NASH mice and patients [88]. These liver-resident T cells were reprogrammed and activated by metabolic stimuli, mediating liver damage [88]. NASH-HCC was found to have a worse outcome in patients treated with PDL1/PD-1 immunotherapy due to expansion of activated CD8$^+$ killer T cells [89]. These findings highlights a potential role of activated CD8$^+$ T cells in HCC progression, which has implications for immunotherapy [89]. Leslie et al. showed in preclinical NASH-HCC models that antagonism of CXCR2, a chemokine receptor that is exclusively expressed on neutrophils in mice and humans, resulted in efficient tumour clearance and increased survival when combined with anti-PD-1 blockade [90]. This work demonstrated that sensitization of NASH-HCC may be beneficial to improve the efficacy of systemic treatments.

DNA methylation, oxidative stress and hepatocyte reprogramming

DNA methylation

NAFLD and T2D are multifactorial diseases influenced by hereditary genetics and environmental factors, which can induce hepatic epigenetic alterations [91]. In NAFLD patients, epigenetic changes are known to promote liver fibrosis [92]. Thus, DNA methylation can occur in tissues undergoing metabolic reprogramming, which involves pathways such as insulin signalling and secretion, adipocyte differentiation, mitochondrial function, lipid and glucose homeostasis, and inflammation [93].

DNA methylation signatures have also been identified in NASH patients [94]. NASH hepatic methylation can be reversible, as seen in liver biopsies of obese subjects who underwent bariatric surgery to lose weight [95]. Interestingly, a DNA methylation

signature obtained from the peripheral blood of NASH patients showed epigenetic age acceleration correlating with increased liver fibrosis [96]. Whether this DNA methylation profile found in NASH patients is related to HCC development requires further research.

In a large-scale, multi-omics study of HCC patients, alterations by hypermethylation and mutation were observed in metabolic reprogramming genes [97]. Notably, *CPS1*, a urea cycle enzyme, was found to be hypermethylated in HCC and correlated with a reduction in *CPS1* mRNA levels [97]. *CPS1* deficiency induced excess ammonia and activated fatty acid oxidation, which provides ATP for proliferation in HCC cells [98]. The methylation profile was also analysed to identify differentially methylated genes in T2D and HCC, among those *CDKN1A* was found as a potential diagnostic and prognostic marker in HCC [99]. Additional studies in large patient's cohorts investigating the DNA methylation profile in patients are required to understand the potential role of epigenetic modifications related to NAFLD/T2D in the development of HCC.

Oxidative Stress

NAFLD and T2D are characterized by an increase in hepatic fat accumulation and chronic low-grade inflammation, which induces an excessive amount of reactive oxygen species (ROS). This increase in ROS leads to oxidative stress and liver damage which has been strongly implicated in HCC (**Fig. 2**). The effect of oxidative stress in obesity and HCC was covered extensively in our recent review [31].

Mitochondria are involved in ROS production through their activity in energy metabolism and oxidative phosphorylation. Thus, oxidative stress induced by dysfunctional mitochondrial activity in obesity has been identified as a driver of liver

pathophysiology. Using high-resolution respirometry, mitochondrial respiration was quantified in liver biopsies of obese, insulin-resistant patients, with or without NAFLD/NASH, and compared to lean/healthy patients [100]. Hepatic mitochondrial respiration rate was higher in obese, insulin-resistant patients compared to lean controls [100]. However, among patients with obesity and insulin resistance, those with NASH were found to have a lower hepatic respiration rate compared to those without NASH. Furthermore, the patients with NASH had significantly increased hepatic insulin resistance, hepatic oxidative stress, and inflammation [100]. Loss of this increased hepatic mitochondrial respiration in NASH patients results in elevated oxidative stress, driving disease progression to HCC [31].

Protein tyrosine phosphatases (PTPs) are a protein family that has been identified as a key regulator in oxidative stress and insulin resistance. PTPs contain a catalytic cysteine in their phosphatase domain that is highly susceptible to oxidation by ROS. We demonstrated that a HFD induced oxidative stress in obesity, which led to prominent PTP oxidation in the liver [42]. PTPN2 (TCPTP) was inactivated increasing lipogenesis and insulin-STAT5 signalling. This enhanced expression of STAT5 promoted IGF-1 production in the liver, increasing insulin resistance and the progression to T2D [42]. Moreover, PTPN2 inactivation in the liver contributes to NASH and HCC development through STAT1 and STAT3-dependent mechanisms, respectively [78].

Oxygen availability, oxidative stress, inflammation, and various nutrients can differentially affect hepatocyte signalling between the portal and central vein. Importantly, liver zonation can affect metabolic reprogramming in various hepatic regions [32]. Although oxidative stress promotes cancer development, tumour cells can also utilize antioxidant systems for survival. Thioredoxin reductase-1 (TrxR1), an

antioxidant protein, was found to be significantly overexpressed in human HCC samples [101]. NRF-2, a key transcription factor in oxidative stress regulation, was shown to upregulate TrxR1 expression in HCC cell lines [101]. Treating with a TrxR1 inhibitor *in vitro* and *in vivo* exhibited potent anti-tumour effects and increased sensitivity to sorafenib treatment. Taken together, these studies highlight the potential oncogenic role of antioxidant systems in HCC, which may guide better treatment options for patients with a high antioxidant profile.

Metabolic driven hepatocyte reprogramming

An increase in insulinemia, hepatic gluconeogenesis, and lipogenesis, with excessive lipid accumulation, represent the hallmarks of NAFLD and T2D have been found to alter hepatocyte function (**Fig. 3**). In obesity, fasting insulin concentration and insulin secretion are increased in response to meals. On average healthy individuals have 60 nmol of insulin 4h after a meal, whereas obese individuals can reach more than 140 nmol of insulin in the same conditions [102]. This hyperinsulinemia associated with obesity increases cancer mortality, including HCC [103]. Insulin signalling modulates proliferation, survival and differentiation through RAS, AKT and PI3K, which are frequently mutated genes in HCC and are considered therapeutic targets [104]. Moreover, PTEN, a well-known HCC tumour suppressor, negatively regulates insulin signalling and is also frequently mutated in liver cancers [105].

In a streptozotocin-induced diabetic rodent model, transplanted pancreatic islets of Langerhans induced hepatocellular neoplasms [106]. This method for treating T1D involved transplanting functional islets in the liver via the portal vein. However, the rats developed liver tumours which may have been a consequence of increased insulin secretion and subsequent growth stimulation from the transplanted islets [106].

Mature hepatocytes maintain plasticity through Hedgehog, Hippo-YAP-TAZ and Notch, which are activated during obesity and hyperinsulinemia to cope with chronic insults [107, 108]. Notch-mediated signalling can reprogram hepatocytes to cholangiocytes or progenitors in chronic liver injury [107]. Notch overexpression in mature mouse hepatocytes led to the expression of biliary markers SOX9 and osteoponin, which are normally absent in hepatocytes. Consistently, mice fed with a methionine- and choline-deficient diet (a NASH mouse model) resulted in SOX9 induction, steatohepatitis and biliary trans-differentiation [107]. Loss of hepatocyte identity plays a role in the transformation process into cancer cells and in dedifferentiation into precursor cells that can later develop into malignant cells. Proteomics data from mouse NASH livers revealed a downregulation in hepatocyte identity genes, suggesting their importance in disease progression [109]. The involvement of ELF3 and GLIS2 was found to play a role in NASH [109]. These transcription factors regulate the activation of hepatokines, such as Spp1 and Ctgf, which regulate the crosstalk between hepatic cells to induce NASH progression. Spp1 and Ctgt likely contribute to the activation of hepatic stellate cells and fibrosis [109]. Stellate cells function as a signalling hub and secrete growth factors, cytokines, and chemokines named “stellakines”. These secreted factors have been found to be increased in NASH, suggesting their contribution to disease progression and HCC development [32].

Hyperglycaemia can provide additional “fuel” to cancer cells to maintain their fast proliferation state (**Fig. 3**). Glucose metabolism was investigated in non-transformed livers from mice on a short-term HFD [45], showing an increase in glucose uptake by 35%. Mice had an increase in lactate production, which recapitulated the high lactate phenotype in obese patients. The tricarboxylic (TCA) cycle is central for energy

metabolism (**Fig. 3**). Glycolysis-derived pyruvate can enter the TCA cycle after being converted into acetyl-CoA, or as oxaloacetate through pyruvate carboxylase (PC). Mice fed with an HFD had increased levels of hepatic pyruvate, malate and citrate [45]. HFD increased the pentose phosphate pathway and serine biosynthesis, as well as PC activity, suggesting that a fat-rich diet could induce an increase in glucose uptake similar to the tumoral state [45]. Indeed, HFD intake increased liver tumours in DEN-injected mice [45]. Serine biosynthesis and mitochondrial PC-activity were elevated in HCC tissue from DEN-injected mice compared to liver tissue of control diet mice [45]. In summary, obesity and T2D can increase cancer hallmarks in non-transformed livers suggesting that hyperinsulinemia, dysregulated glucose homeostasis and an increase in lipids can activate pathways that promote hepatic tumour development.

Novel disease models and therapeutic possibilities

Stem cell differentiation and somatic cell reprogramming to mimic human pathology

The connection between NAFLD/T2D and HCC requires novel methods for understanding their pathophysiology. Studies in liver disease have mainly relied on human tissue/biopsies from donors, animal models, *in vitro* cell lines, and cultured primary hepatocytes. However, all these models have major limitations.

One recent development in modelling human liver function is induced pluripotent stem cells (iPSCs) [110-112]. iPSCs can be derived from somatic cells of subjects with different pathologies, including HCC patients with or without diabetes. iPSCs are then differentiated into hepatocyte-like cells (HLCs), which can be expanded and maintained in culture [113]. They do not de-differentiate and maintain genomic and physiological similarities to human hepatocytes. Indeed, biobanking of patient iPSCs

has aided in the acceleration of precision medicine. These patient-derived stem cell models can be used to predict a patient's response to drug treatments.

Several studies have developed methods to differentiate human iPSCs to a hepatic cell fate. This differentiation protocol requires various growth factors such as Activin A, FGF2, BMP4, HGF, and OSM, and specific culture conditions to generate mature hepatocytes [110]. Moreover, a hepatic-like phenotype can be achieved in somatic cells via the ectopic expression of native liver-enriched transcription, bypassing the intermediate pluripotent state [114]. These "artificial" hepatocytes are amenable to CRISPR/Cas gene editing and useful for large-scale high-throughput screening and toxicology studies.

Organoids

While the advancement of iPSC-derived HLCs has shown promise as an improved model of hepatocyte function, this method has been criticized due to the monolayer cell culture condition. Recent developments have demonstrated that 3D cell culture can more accurately simulate the cell's environment by allowing cell-cell, cell-extracellular matrix (ECM), and mechanical interactions [115]. ECM components such as collagen, laminin, and fibronectin have been implemented in 3D culture to mimic the liver microenvironment. 3D culture methods have been developed using synthetic scaffolds or by spontaneous hepatic organoids. Moreover, co-culture of HLCs with other cell types also allows for a better model of liver physiology.

Steatosis can be induced in iPSC-derived human liver organoids with free fatty acid treatment [116]. Liver organoids treated with antidiabetic drugs, L-carnitine and metformin, showed improvement in fat accumulation [116]. Organoids have also been

generated from patient liver cancer cells to investigate their use as models in HCC [117]. Liver cancer organoids were found to reflect patient-specific histological architecture and gene expression, furthermore, they developed tumours when transplanted *in vivo* [117].

The use of iPSCs and organoids has great potential in the advancement of personalized medicine for NAFLD and T2D. Additionally, genomic analysis of human iPSCs and organoids can identify genetic variants that may confer drug resistance or diagnostic biomarkers for disease. iPSCs and organoids have been proposed as important tools in regenerative medicine. Using gene editing, these *in vitro* models can be developed to repair mutations in genetic diseases and transplanted in patients. Moreover, iPSCs and organoids can be genetically modified to be HLA-matched to patients which prevents organ rejection. Although iPSCs and organoids have limitations, such as high cost and poor reproducibility, there is great promise in this technology to advance research and develop effective treatments.

Pharmacological therapies and clinical trials

The dramatically rising incidence of NAFLD, T2D and HCC has prompted the need for effective therapeutic options for patients. Given that T2D is a known risk factor for HCC, several studies have investigated antidiabetic treatments for liver cancer. Increasing evidence suggests that diabetic agents may also be attractive therapies and play a relevant role in management for HCC patients. The effects of antidiabetic drugs in HCC can be evaluated at two main levels: chemoprevention and treatment (**Table 1**).

Sorafenib was the first systemic therapy that was found to be effective in an advanced HCC clinical trial [118]. For a decade, it was the only approved first-line treatment for HCC patients. Recently, several new effective treatments have been approved for first-line therapies and second-line therapies [118-123]. These include several kinase inhibitors, VEGF inhibitors, and immune-checkpoint inhibitors. However, there is still a concern for adverse events associated with systemic therapies. Developing alternative strategies to improve patient's quality of life will be crucial in the advancement of HCC treatments.

Given the role of lipogenesis in NAFLD-associated HCC, FASN inhibitors have also bene proposed as promising treatments. As mentioned previously, FASN was found to inhibit HCC formation in oncogenic mouse models [46, 47]. In a preclinical study, FASN inhibitors were found to improve efficacy in combination HCC treatments [124]. Additionally, the use of FASN inhibitor (TVB-2640) in a NASH clinical trial showed efficacy in decreasing liver fat and improving biochemical biomarkers [125]. These findings provide support for investigating FASN inhibitors in NAFLD-associated HCC.

The number of patients with diabetes is generally not specified in clinical trials (**Table 1**). In three studies in second-line drugs, diabetic patients are indirectly referred to as subgroups with NASH [121-123]. The classification of therapeutic groups should consider both the presence of diabetes and the level of metabolic control. This could optimize the efficacy of systemic treatments.

Metformin is an insulin sensitizer that reduces hepatic gluconeogenesis and hyperinsulinemia. It activates the AMPK pathway via inhibition of mitochondrial respiration, which increases insulin sensitivity [126]. Activation of AMPK also leads to downstream inhibition of mTOR, which plays a key role in proliferation and immune

activation in cancer. In HCC patients with T2D, metformin treatment prolonged overall survival [127]. Metformin was found to reduce HCC risk in a network meta-analysis of clinical studies (**Table 1**) [128-130]. It should be considered that metformin was also associated with a poor response to sorafenib treatment in HCC patients [127, 131, 132], but opposite results have also been reported [133-135].

Thiazolidinediones (TZDs) are another class of antidiabetic drugs that activate PPARs, key regulators in glucose metabolism and insulin sensitivity [136]. These drugs have also been identified for their anti-tumoral role and are involved in cell growth arrest, apoptosis induction, and preventing cell invasion. In liver cancer, TZD agents pioglitazone and rosiglitazone were associated with a reduction in liver cancer incidence in T2D patients [136]. Like metformin, TZDs were found to reduce HCC risk [127, 137]. However, TZDs have also been linked to an increased risk of cardiovascular events in patients with cirrhosis [138]. Further studies with TZDs as a treatment for HCC patients with T2D are required to determine its potential efficacy.

Several other classes of antidiabetic drugs have been proposed as potential therapeutics in HCC (**Table 1**). A recent epidemiological study found that SGLT2 inhibitors were associated with improved overall survival in HCC patients with T2D [139]. GLP-1RAs have also been investigated *in vitro* and *in vivo* in HCC models. However, large scale studies on patients are required to determine the beneficial effects of these drugs.

Future directions

Understanding the complexity of the pathogenic pathways involved in NAFLD/T2D-related HCC remains to be elucidated. Determining the etiopathogenetic factors of T2D in both NAFLD and non-NAFLD scenarios will impact the understanding of HCC initiation and progression (i.e., patients with T2D without NAFLD but with an active

virus infection in hepatitis C or B, and non-abusive alcohol consumption). Recently, it has been recommended that NAFLD should be replaced by metabolic dysfunction-associated fatty liver disease (MAFLD) to downplay the importance of alcohol in the definition of NAFLD and to emphasize the metabolic risk factors that underlie progression of NAFLD-associated pathology [140, 141]. Considering this non-exclusive diagnosis, the development of HCC should be also analysed based on the response to changes in diet and anti-diabetogenic drug treatment.

Mouse models cannot fully recapitulate mechanisms of human disease progression, as was observed in a comparative study between NAFLD/NASH patients and experimental mice [142]. New disease models and therapeutic treatments can aid in the understanding of T2D as a risk factor for HCC. The development of 3D organoids derived from induced pluripotent stem cells or organoids derived from cancer cells of diabetic patients that can recapitulate human genetic expression will provide further understanding of disease biology. New emerging technologies, including single-cell RNA sequencing, spatial transcriptomics, and advanced metabolomics, are also promising for studying hepatic cell networks, cellular heterogeneity, and cancer clonal evolution [143-145]. These single-cell technologies will deliver new insights into disease-associated reprogramming and further our understanding of the pathological mechanisms linking NAFLD/T2D and HCC.

Conclusions

Dietary intake is crucial in the maintenance of metabolic health. Increased dietary fat and simple sugars are major inducers of altered metabolism, which includes increased lipogenesis, lipid accumulation and insulin resistance. The key responsible regulators usually involve transcription factors or upstream components controlling lipid

metabolism. Obesity, NAFLD and T2D promote liver inflammation and increase oxidative stress, which accelerates oxidative cell death and promotes HCC. Therefore, HCC shares common altered metabolic pathways with NAFLD/T2D, suggesting the involvement of dysregulated lipidaemia and insulinemia in tumorigenesis promotion. The molecular pathways leading to the transition from a high-fat, insulin-resistant, inflammatory liver to tumorigenesis are not well understood. Yet some of the emerging key players have been described in this review, highlighting our understanding of NAFLD/T2D-associated HCC so far. The use of innovative technologies such as 3D organoids will increase our understanding of these disease and reveal an overview of novel therapeutic targets.

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Author contribution

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Conflict of interest

The authors declare no conflict of interest.

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Figure Legends

***Figure 1*. Obesity and overnutrition expose the liver to an overload of energetic fuels (lipids, carbohydrates and proteins) that can affect hepatic energy metabolism with pathological consequences.** Several transcription factors are known to act as sensors of nutrients, such as PPAR-γ, which is activated by lipophilic ligands such as PUFAs; SREBP1c, which is activated by LXR in response to insulin, PUFAs and oxysterols; ChREBP, which is activated by glucose-6-phosphate; or mTORC1, which is activated in response to amino acids. These and other transcription factors regulate the expression of enzymes and signalling proteins required to execute and coordinate major energy metabolic pathways. The consequence of chronic aberrant activation of these transcription factors, associated with hyperinsulinemia during T2D predispose the liver to steatosis, inflammation, fibrosis, oxidative stress and mitochondrial dysfunction. These factors facilitate oncogenic transformation and HCC development. Selective insulin resistance confers liver resistance to the inhibitory action of insulin on gluconeogenesis, while the sensitivity of the liver to the stimulatory effect of insulin over lipogenesis remains. Different types of nutrients provided by the diet can accelerate metabolic dysfunction, including nutrients that are abundant in industrialised highly palatable and caloric foods such as saturated fat, cholesterol sucrose and fructose.

***Figure 2.* NAFLD and T2D are characterized by increased hepatic inflammation and oxidative stress, contributing to HCC development.** The population of resident

and recruited immune cells in the liver is dynamic during the progression of NASH. Different cell types can participate in the inflammatory response. Pro-inflammatory cytokines such as IL-6 and TNF-α, are released by different tissue sources and drive an inflammatory response, including oncogenic STAT3 activation. An excess of dietary fats and simple carbohydrates favour body and hepatic fat accumulation and alters specific immune cell populations in the liver. Tumour suppressive $CD4^+$ T cells are depleted by nutrient overload and ROS-dependent mechanisms. Hepatic resident Kupffer cells (resKCs) are also depleted in NAFLD and replaced by two subsets of pro-inflammatory recruited macrophages: monocyte-derived KCs and hepatic lipid-associated macrophages (LAMs). These macrophages co-localize with fibrotic liver regions with activated hepatic stellate cells. In association with hepatic steatosis and inflammation, continuously high ROS levels lead to oxidative stress and liver damage that is strongly associated with HCC development.

Figure 3. Hepatocyte transformation in NAFLD/T2D. Normal hepatocytes prefer β-oxidation of fatty acids as a source of energy, as well as relatively low glucose uptake and oxidation. This is especially true during fasting conditions and this preference is influenced by hepatic zonation. Transformed hepatocytes exhibit high glucose uptake and rely on aerobic glycolysis as a source of energy. Pyruvate is preferentially converted into lactate, instead of being oxidised in the mitochondria. High-fat intake rewires hepatocyte energy metabolism to favour glucose uptake and its utilization as a source of energy through aerobic glycolysis and lactate production. Glucose can be used as a carbon source for biosynthetic reactions in rapidly growing tissues, as well as in cell signalling and redox state. High glucose uptake also sustains the substrate requirement for the pentose phosphate pathway. This is important for ribulose-5-phosphate synthesis, which is required for nucleotide biosynthesis and nucleic acid

replication. Pyruvate carboxylase is induced by high-fat intake, favouring the entrance of pyruvate in the TCA cycle as oxaloacetate to maintain anaplerotic reactions required for amino acid biosynthesis. Acetyl-CoA is converted into fatty acids through lipogenesis. The pool of intracellular fatty acids (from lipogenesis and extrahepatic tissues) is used for phospholipid biosynthesis to build biological membranes. Hyperinsulinemia associated with T2D, in combination with the action of the hepatokine IGF-1, can lead to dysfunctional signalling pathways involved in cell survival, apoptosis, and stress response. Together, these cellular and metabolic changes can represent advantages for cancer cells, allowing them to sustain rapid growth and proliferation.

Tables

Pharmacological therapies and clinical trials										
Chemoprevention										
Study	**Phase/Type**	**Identifier**	**Number of patients**	**Study drug**	**Diabetes mellitus**	**Number centres/ countries**	**Year**	**Primary end-point**	**Conclusions**	**Reference**
Evans JM *et al.*	Retrospective case-control study	N/A	Cases (983) – Controls (1846)	Metformin	T2D	Population databases/Tayside -Scotland	2005	Odds Ratio	Metformin may reduce the risk of cancer in patients with T2D. The unadjusted odds ratio was 0.86 (95% confidence interval 0.73 to 1.02). The unadjusted odds ratio for any exposure to metformin since 1993 was 0.79 (0.67 to 0.93).	[146]
ADOPT	III	NCT00279045	Rosiglitazone (1456) vs Metformin (1454) vs Glyburide/Glibenclamide (1441)	Metformin, TZD, sulfonylurea	T2D	490 centres/U.S.A., Europe/hospital based	2006	Monotherapy failure	Post-hoc analysis of occurrence of HCC in patients enrolled in OADM monotherapy trial. HCC total: 4. HR metformin vs rosiglitazone: 0.92 (95% CI 0.63–1.35); metformin vs glibenclamide: 0.78 (95% CI 0.53–1.14)	[147]
RECORD	III	NCT00379769	Rosiglitazone (2220) vs Metformin (1122) vs Sulfonylurea (1105)	Metformin, TZD, sulfonylurea	T2D	447 centres/U.S.A., Australia/ hospital based	2007	Cardiac outcomes, regulation of glycaemia	Post-hoc analysis of HCC incidence in cardiovascular outcomes study of OADM, with addition of another " rescue" OADM as needed for glycaemic control. HCC total: 4. On background of sulfonylurea: metformin vs rosiglitazone HR 1.22 (95% CI 0.86–1.74). On background of metformin: sulfonylurea vs rosiglitazone HR 1.33 (95% CI 0.94–1.88)	[100]
Donadon V *et al.*	Retrospective case-control study	N/A	HCC cases (610) - Matched liver cirrhosis (618) - Controls (1696)	Metformin	T2D	1 centre /Italy	2010	To explore the relationships among T2D, antidiabetic therapy and HCC risk. Odds Ratio	T2D is an independent risk factor for HCC and pre-exists to HCC occurrence. Metformin was associated with a significant reduction of risk for HCC vs controls vs liver cirrhosis cases when compared with sulfonylurea and insulin therapy (OR of 0.15; CI 0.04–0.50; p= 0.005 and OR = 0.16; CI 0.06–0.46; p= 0.0006 respectively)	[148]
Chang CH *et al.*	Retrospective case-control study	N/A	T2D (606,583). A total of 10,741 liver cancer cases, 7,200 colorectal cancer cases, and 70,559 diabetic controls were included.	TZD	T2D	Population databases /Taiwan	2012	To assess the association between TZDs (both pioglitazone and rosiglitazone) and the occurrences of liver, colorectal, lung, and urinary bladder cancers.	The use of pioglitazone and rosiglitazone is associated with a decreased liver cancer incidence in diabetic patients. A significantly lower risk of liver cancer incidence was found for any use of rosiglitazone (OR: 0.73, 95% CI: 0.65-0.81) or pioglitazone (OR: 0.83, 95% CI: 0.72-0.95), respectively.	[136]
Singh S *et al.*	Meta-analysis	N/A	Ten studies reporting 22,650 cases of HCC in 334,307 patients	Metformin, TZD, sulfonylurea, insulin	T2D	Multicentre	2013	Risk of incident HCC	Meta-analysis of observational studies showed a 50 % reduction in HCC incidence with metformin use, but an increase in HCC incidence with sulfonylurea or insulin use. TZD did not modify the risk of HCC.	[128]
Zhang H *et al.*	Meta-analysis	N/A	Seven studies reporting 562 cases of HCC in 16,549 patients	Metformin	T2D	Population databases/China	2013	To determine the association between metformin use and HCC among diabetic patients.	Metformin treatment was associated with reduced risk of HCC in diabetic patients (relative risk (RR) 0.24, 95% confidence interval (CI) 0.13–0.46, p < 0.001).	[129]

Lai SW *et al.*	Retrospective cohort study	N/A	Controls diabetics on TZD treatment (23580)/ Case diabetics on TZD treatment (23580)	TZD	T2D	Taiwan	2020	Risk of incident HCC	There was a negative association in a duration-dependent manner between the risk of HCC and TZD use among T2D patients who had risk factors for HCC	[137]
Vilar-Gomez E *et al.*	Cohort	N/A	No T2D (87) vs T2D (212)	Metformin, sulfonylurea, insulin	T2D and NASH cirrhosis	6 centres/Europe, Asia, Australia, Cuba	2021	To determine the influence of T2D, hyperglycaemia, and ADMs on outcomes of HCC, liver decompensation, and death	Metformin significantly reduced the risk of hepatic decompensation and HCC only in subjects with HbA1c levels greater than 7.0% (aHR,0.97; 95% CI, 0.95–0.99 and aHR, 0.67; 95% CI, 0.43–0.94, respectively)	[149]
Kaplan DE *et al.*	Retrospective cohort study	N/A	74 984 diabetics; 40,368 with T2D before cirrhosis. 11 114 had active utilization of metformin.	Metformin	T2D	Population databases/U.S.A.	2021	To investigate the impact of metformin exposure on mortality, hepatic decompensation, and HCC in individuals diagnosed with cirrhosis with a pre-existing diagnosis of diabetes mellitus	Metformin use in patients with cirrhosis and diabetes appears safe and is associated independently with reduced overall, but not liver-related, mortality, hepatocellular carcinoma, or decompensation after adjusting for concomitant statin and angiotensinogen-converting enzyme inhibitor/angiotensin-2–receptor blocker exposure.	[150]
Yen FS *et al.*	Observational case-control study	N/A	2828 paired propensity score matched DPP-4 inhibitor users and nonusers T2D with compensated liver cirrhosis	Dipeptidyl peptidase-4 (DPP-4) inhibitors	T2D	Population databases/Taiwan	2021	To assess the outcomes of all-cause mortality, HCC, major adverse cardiovascular events (MACEs), decompensated cirrhosis, and hepatic failure.	DPP-4 inhibitor users were associated with higher risks of decompensated cirrhosis and hepatic failure than did nonusers among patients with T2D and compensated liver cirrhosis. Risk of all-cause mortality, HCC, and major cardiovascular events between DPP-4 inhibitor users and nonusers were not statistically different.	[151]
Li Q *et al.*	Meta-analysis	N/A	Seven studies reporting 562 cases of HCC in 16,549 patients	Metformin	T2D	Multicentre	2022	To evaluate the relationship between metformin therapy and HCC survival and risk.	Metformin in T2D patients is significantly associated with reduced risk and all-cause mortality of HCC (OR/RR = 0.59, 95% CI 0.51 0.68, I2 = 96.5%, p < 0.001).	[130]
Kramer JR *et al.*	Cohort	N/A	T2D and NAFLD (85963)	Metformin, sulfonylurea, insulin	T2D and NAFLD	130 centres/U.S.A.	2022	Risk of incident HCC	Use of metformin was associated with a reduced risk of HCC compared with no medication, 22% lower risk of HCC (HR, 0.77; 95% CI, 0.65–0.90; p = 0.001), whereas use of combination therapy was associated with increased risk (HR for insulin and metformin, 1.53; 95% CI, 1.26–1.86; p < 0.0001; HR for insulin, metformin, and sulfonylureas, 1.71; 95% CI, 1.41–2.08; p < 0.0001).	[152]
Hendryx M et al.	Retrospective cohort study	N/A	3,185 HCC patients with pre-existing diabetes, 137 (4.3%) patients used SGLT2 inhibitors.	Sodium-glucose cotransporter 2 (SGLT2) inhibitors	T2D	SEER-Medicare dataset/U.S.A.	2022	Adjusted hazard ratios for mortality	SGLT2 inhibitor initiation was associated with improved overall survival of HCC patients with pre-existing type 2 diabetes compared with no SGLT2 inhibitor use (HR = 0.68, 95% CI = 0.54–0.86).	[139]
Treatment HCC										
Study	**Phase/Type**	**Identifier**	**Drug (n)**	**Study drug**	**Diabetes mellitus**	**Number centres/ countries**	**Year**	**Primary end-point**	**Conclusions**	**Reference**
Chen TM *et al.*	Retrospective cohort study	N/A	No T2D RFA (82) / T2D RFA with metformin (21)/ T2D without metformin (32)	Metformin, sulfonylurea, insulin	T2D 32,3%	1 centre/Taiwan	2011	OS	Metformin users among T2D patients with HCC undergoing RFA had a favourable overall survival compared with T2D patients without metformin treatment	[153]
Bhat M *et al.*	Retrospective cohort study	N/A	No T2D (438)/T2D not on metformin (207)/T2D on metformin (56)	Metformin	T2D 37,5%	1 centre (part BRIGDE COHORT)	2014	OS	This study demonstrates no survival benefit to the use of metformin in T2D patients with HCC	[154]
Jang WI *et al.*	Retrospective cohort study	N/A	SBRT without T2D (169) /SBRT T2D not on metformin (29) / SBRT T2D on metformin (19)	Metformin	T2D 22%	4 centres/Korea	2015	OS	The use of metformin in patients with HCC receiving radiotherapy was associated with higher overall survival. In the propensity score-matched cohort (n=76), the OS rate of the metformin group was higher than that of the non-metformin group (2-year, 76% vs. 37%, p=0.022).	[155]
Seo YS *et al.*	Retrospective cohort study	N/A	Curative resection T2D on metformin (533)/ Curative resection T2D not on metformin (218)	Metformin	T2D	National database (NHIS and KKRC)/Korea	2016	OS	In patients treated with curative hepatic resection, metformin use was associated with improvement of HCC-specific mortality and reduced occurrence of retreatment events.	[156]
Casadei Gardini A *et al.*	Retrospective cohort study	N/A	Sorafenib (51) vs Sorafenib+metformin (31) vs Sorafenib+insulin (11)	Multiple kinase inhibitor, metformin, insulin	T2D 42,5%	1 centre/Italy	2015	PFS, OS	The result of greater tumour aggressiveness is described and resistance to sorafenib in patients treated with metformin	[132]
Casadei Gardini A *et al.*	Prospective cohort study	N/A	Sorafenib (193) vs Sorafenib+metformin (52) vs Sorafenib+insulin (34)	Multiple kinase inhibitor, metformin, insulin	T2D 30,0%	1 centre/Italy	2017	PFS, OS	In HCC patients undergoing chronic treatment with metformin, the use of sorafenib was associated with poor PFS and OS (1.9 and 6.6 months, respectively) compared to 3.7months and 10.8 months, respectively, for patients without T2D and 8.4 months and 16.6 months, respectively, for patients on insulin (P < .0001).	[131]

Chung YK *et al.*	Retrospective case-control study	N/A	Recurrence after LR: Sorafenib+metformin (40)/ Sorafenib+insulin (23)/ Sorafenib control (241); propensity score matching control (40) Recurrence after LT: Sorafenib+metformin (14) /Sorafenib+insulin (17) / Sorafenib control (43); propensity score matching (28)	Multiple kinase inhibitor, metformin, insulin	T2D	1 centre/Korea	2018	OS	Absence of synergistic antitumor effects of metformin.	[133]
Schulte L *et al.*	Retrospective case-control study	N/A	5093 patients with HCC, 1917 patients (37.6%) were diagnosed with T2D, of which 338 (17.6%) received treatment with metformin	Metformin	T2D (37,6%)	3 centres/Germany, Austria	2019	OS	In the matched cohorts, mOS remained significantly longer in metformin-treated patients (22 vs 16 months, P = 0.021). Co-treatment of metformin and sorafenib was associated with a survival disadvantage.	[127]
El Shorbagy S *et al.*	RCT	N/A	Sorafenib+metformin (40) vs sorafenib (40)	Multiple kinase inhibitor, metformin	T2D 60%	2 centres/Egypt	2021	OS, TDP, Safety	No superior efficacy of adding metformin to sorafenib in HCC treatment	[134]
Cho YY *et al.*	Retrospective cohort study	N/A	1,566 unresectable HCC patients who received sorafenib. Long-term survivor group (survival more than two years, n = 257) or a control group (n = 1309).	Multiple kinase inhibitor	Presence T2D analysed but percentage by groups not reported	9 centres/Korea	2021	Clinical characteristics of long-term survivors after sorafenib treatment.	The prognostic factors predicting long-term survival were metformin use (adjusted hazard ratio [aHR] = 3.464; P < 0.001), hand-foot skin reaction (aHR = 1.688; P = 0.003), and concomitant treatment with chemoembolization or radiotherapy (aHR = 2.766; P < 0.001).	[135]
Systemic therapy for HCC										
First line										
Study	**Phase**	**Identifier**	**Drug (n)**	**Study drug / Molecular target**	**Diabetes mellitus**	**Number centres/ countries**	**Year**	**Primary end-point**	**Conclusions**	**Reference**
SHARP	III	NCT00105443	Sorafenib (299) vs placebo (303)	Multiple kinase inhibitor: VEGFR, KIT, RET, FLT-3, PDGFR-β, RET/PTC, MAPK	Not specified	178 centres/23 countries	2008	OS	Sorafenib improves survival compared with placebo	[118]
Asia-Pacific	III	NCT00492752	Sorafenib (149) vs placebo (75)	Multiple kinase inhibitor: VEGFR, KIT, RET, FLT-3, PDGFR-β, RET/PTC, MAPK	Not specified	23 centres/China, South Korea and Taiwan	2009	OS	Sorafenib improves survival compared with placebo	[157]
REFLECT	III	NCT01761266	Lenvatinib (478) vs sorafenib (476)	Multiple kinase inhibitor: VEGFR 1-3, FGFR 1–4, PDGFR α, RET, KIT	Not specified	154 centres/24 countries	2018	OS	Lenvatinib is non-inferior compared with sorafenib	[119]
IMbrave 150	III	NCT03434379	Atezolizumab + bevacizumab (336) vs sorafenib (165)	Checkpoint inhibitor + Antiangiogenic: Anti-PD-L1 antibody + Anti VEGFA antibody	Not specified*	111 centres/17 countries	2020	OS, PFS (co-primary)	Atezolizumab plus bevacizumab improve overall survival compared with sorafenib	[120]
HIMALAYA	III	NCT03298451	Durvalumab + tremelimumab (Stride 393) vs sorafenib (389)	Checkpoint inhibitor + checkpoint inhibitor: Anti-PD-1 antibody + Anti-CTLA-4 antibody	Not specified	181 centres/16 countries	2022	OS	Durvalumab plus tremelimumab improve overall survival compared with sorafenib	[158]
COSMIC-312	III	NCT03755791	Atezolizumab + cabozantinib (432) vs sorafenib (217)	Checkpoint inhibitor + multiple kinase inhibitor: Anti-PD-L1 antibody + VEGFR, MET, TAM family receptors (TYRO3, AXL, MER	Not specified*	178 centres/32 countries	2022	PFS, OS (dual)	Atezolizumab plus cabozantinib improve progression-free survival compared with sorafenib	[159]
CheckMate 459	III	NCT02576509	Nivolumab (371) vs sorafenib (372)	Checkpoint inhibitor: Anti-PD-1 antibody	Not specified*	22 countries	2022	OS	Nivolumab does not improve survival compared with sorafenib	[160]
Second line										
RESORCE	III	NCT01774344	Regorafenib (379) vs placebo (194)	Protein kinase inhibitor: RAF-1, RET, BRAFV600E, VEGFR, TIE-2, PDGFR, FGFR, EGFR, CSF1R, c-kit	Not specified. NASH: 25(7%) vs 13(7%)	152 centres/21 countries	2017	OS	Regorafenib improves survival compared with placebo	[121]
CELESTIAL	III	NCT01908426	Cabozantinib (470) vs placebo (237)	Multiple kinase inhibitor: VEGFR, MET, TAM family receptors (TYRO3, AXL, MER)	Not specified. NASH: 43(9%) vs 23(10%)	95 centres/19 countries	2018	OS	Cabozantinib improves survival compared with placebo	[122]

REACH-2	III	NCT02435433	Ramucirumab (197) vs placebo (95)	Monoclonal antibody: Anti VEGFR-2	Not specified. NASH: 19(10%) vs 4(4%)	92 centres/22 countries	2019	OS	Ramucirumab improves survival compared to placebo with AFP ≥ 400 ng/ml	[123]
KEYNOTE-224	II	NCT02702414	Pembrolizumab (104)	Checkpoint inhibitor: Anti-PD-1 antibody	Not specified*	47 centres/10 countries	2018	ORR	Pembrolizumab approved by the US FDA	[161]
CheckMate-040	I/II	NCT01658878	Nivolumab + ipilimumab (140)	Checkpoint inhibitor + checkpoint inhibitor: Anti-PD-1 antibody + Anti-CTLA-4 antibody	Not specified*	31 centres/10 countries	2020	Safety, tolerability, ORR	Nivolumab and ipilimumab approved by the FDA	[162]
KEYNOTE-240	III	NCT02702401	Pembrolizmab (278) vs placebo (135)	Checkpoint inhibitor: Anti-PD-1 antibody	Not specified*	119 centres/29 countries	2020	PFS, OS (co-primary	Pembrolizumab does not improve survival and progression-free survival compared with placebo	[163]
KEYNOTE-394	III	NCT03062358	Pembrolizumab (300) vs placebo (153)	Checkpoint inhibitor: Anti-PD-1 antibody	Not specified	Asia	2022	OS	Pembrolizumab improves survival compared with placebo in Asia	[164]

Table 1. **The effects of antidiabetic drugs in HCC can be evaluated at two main levels: chemoprevention and treatment.** Information on the presence of diabetes mellitus in clinical trials for the treatment of advanced HCC is very scarce. Abbreviations: T2D, type 2 diabetes; N/A, not applicable; OADM, oral antidiabetic medications; OS, overall survival; PFS, progression-free survival; ORR, objective response rate; TDP, time to disease progression; LT, liver transplantation; LR, liver resection; RFA, radiofrequency ablation; SBRT, stereotactic body radiotherapy; TKI, tyrosine kinase inhibitors; ICI, immune checkpoint inhibitors; PD-1, programmed cell death 1; CTLA-4, cytotoxic T-Lymphocyte antigen 4. * Patients with controlled Type 1 Diabetes mellitus who are on an insulin regimen were eligible for the study.