

Stress kinases in the development of liver steatosis and hepatocellular carcinoma



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ABSTRACT

Non-alcoholic fatty liver disease (NAFLD) is an important component of metabolic syndrome and one of the most prevalent liver diseases worldwide. This disorder is closely linked to hepatic insulin resistance, lipotoxicity, and inflammation. Although the mechanisms that cause steatosis and chronic liver injury in NAFLD remain unclear, a key component of this process is the activation of stress-activated kinases (SAPKs), including p38 and JNK in the liver and immune system. This review summarizes findings which indicate that the dysregulation of stress kinases plays a fundamental role in the development of steatosis and are important players in inducing liver fibrosis. To avoid the development of steatohepatitis and liver cancer, SAPK activity must be tightly regulated not only in the hepatocytes but also in other tissues, including cells of the immune system. Possible cellular mechanisms of SAPK actions are discussed.

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Keywords SAPK; JNK; p38; Steatosis; Hepatocarcinoma; Metabolism

1. INTRODUCTION

Metabolism is a complex network whose regulation is highly reliant on the liver, which controls whole-body energy homeostasis. The liver is the principal organ in charge of maintaining normoglycaemia and lipid and protein metabolism. In the postprandial state, insulin secretion from pancreatic β cells drives glycogen synthesis. Insulin also promotes *de novo* lipogenesis (DNL) through esterification of fatty acids with glycerol-3-phosphate to generate triacylglycerides, ceramides, or cholesterol. Triacylglycerides are stored in lipid droplets or secreted as a component of very low-density lipoproteins (VLDL). During fasting, pancreatic α cells secrete glucagon, which drives hepatic glucose production and fatty acid oxidation to generate acetyl-CoA for energy production or ketone bodies. The liver is thus implicated in a range of pathophysiological processes from nonalcoholic fatty liver disease (NAFLD) and insulin resistance to glycogen or lipid storage deficiencies, urea cycle disorders, and peroxisomal disorders. NAFLD is the leading cause of liver dysfunction in the USA and Europe [1]. This disease is thus associated with several chronic pathologies known as metabolic syndrome, which comprises atherosclerosis, cardiovascular disease, hypertension, insulin resistance, diabetes mellitus, and hyperlipidaemia [2]. Excess intrahepatic fat accumulation can have several causes, including NAFLD, alcoholism, chemotherapy, toxicity, and infectious disease [3]. The mechanisms involved in the development of steatosis include increased DNL and fatty acid flux to the liver, as well as reduced β -oxidation and VLDL secretion [4]. The main cause of an increase in lipogenesis substrates is insulin resistance [5].

In 20%–30% of patients, NAFLD progresses to non-alcoholic steatohepatitis (NASH) [6,7], an observation that led to the two hits model for NAFLD development [8]. The first hit is based on metabolic changes that initiate steatosis, inducing triglyceride accumulation. The second hit causes progression of the pathology, with oxidative stress being a critical factor. However, the two hits model has been discarded because it cannot explain all the molecular changes observed during NAFLD. The disease is instead believed to result from multiple factors acting in parallel on genetically predisposed individuals. Contributing factors include insulin resistance, adipokines, nutritional factors, the gut microbiota, and genetic and epigenetic factors and are included in the multiple hit hypothesis [9]. Prolonged hepatic lipid accumulation has clinical implications because it progresses to NASH, advanced fibrosis, cirrhosis, and liver failure [1]. The analysis of this process is highly complicated, in part because of the election of the appropriate animal model to achieve the research purpose (Table 1). The prevalence of these conditions increases each year, but the molecular mechanisms controlling these pathologies remain poorly understood. The liver has several metabolic and immunological functions that are indispensable for life. The location of the liver ensures its continual exposure to incoming nutrients, products of the intestinal microbiota, and toxic substances [10]. The liver detoxifies metabolites, synthesises essential proteins, and recycles iron from red blood cells [11]. This versatility underpins the fundamental importance of the liver for a healthy physiological state. The liver contains two major cell fractions: parenchymal cells, including the hepatocytes (60%–70% of liver cells) and the cholangiocytes, the nonparenchymal fraction, comprising liver sinusoidal endothelial cells and hepatic stellate cells. Each of these cell

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types has unique functions that contribute to the cooperative regulation of liver function. The liver additionally contains many immune cells involved in the maintenance of homeostasis and the adaptation to hepatic injury, playing key roles in the initiation and progression of liver diseases. The liver immune cell population includes a high density of myeloid cells such as liver-specific macrophages, known as Kupffer cells (KCs), monocyte-derived macrophages, neutrophils, and dendritic cells (DCs). The liver also contains innate lymphoid cells, including natural killer (NK) cells, natural killer T (NKT) cells, $\gamma\delta$ T cells, and mucosal-associated invariant T cells, as well as other T cells and B cells [12–16].

2. STRESS-ACTIVATED KINASE (SAPK) FAMILY

Mitogen-activated protein kinases (MAPK) transduce various extracellular signals that regulate cell proliferation, differentiation, and apoptosis [17] and are implicated in the correct regulation of metabolism. MAPK cascades are triple kinase pathways, including an MKKK (MAPK kinase kinase), a MKK (MAPK kinase), and a terminal MAPK, ensuring signal amplification and fidelity [18]. There are three major groups: extracellular signal-regulated kinases (ERK1/2), c-Jun N-terminal kinases (JNK), and p38 MAPKs. ERKs are mainly activated by mitogens, and JNK and p38 kinases are activated by stress and classified together as stress-

activated protein kinases (SAPK) [17]. SAPKs are activated upon dual phosphorylation of tyrosine and threonine residues in a conserved Thr-X-Tyr loop sequence, in which X is proline in JNKs and glycine in p38 kinases [19]. The activation of JNK is described as mediated by MKK4/7 and the activation of p38 by MKK3/6. The JNK family includes three members encoded by distinct genes. JNK1 and JNK2 are ubiquitously expressed; JNK3 is expressed in brain, testis, and pancreatic β -cells [17]. The p38 family has four isoforms encoded by distinct genes located tandemly in two chromosomes: p38 α (MAPK14) and p38 β (MAPK11), and p38 γ (MAPK12) and p38 δ (MAPK13) [20]. p38 α is ubiquitously expressed, although its expression is lower in brain, liver, and pancreas. p38 β is abundant in the brain, thymus, and spleen; its expression is lower than that in the adrenal glands, lung, kidney, liver, pancreas, and heart; it is not expressed in skeletal muscle. p38 γ is very abundant in skeletal muscle, and p38 δ is highly expressed in pancreas, intestine, adrenal gland, kidney, and heart [21]. Therefore, SAPK activity has been analysed in several tissues, such as heart [22], central nervous system [23], and adipose [24–26], associated with steatosis and liver cancer development. However, although the role of JNKs in liver metabolism and their relation to liver steatosis have been studied in depth [27], less is known of the function of p38 kinases in the control of liver metabolism [26] (Tables 2 and 3).

Table 1 — Animal models of nonalcoholic fatty liver disease (NAFLD) and its progression to nonalcoholic steatohepatitis (NASH), fibrosis, and finally, hepatocarcinoma (HCC).

Model	Diet composition (kcal %)	Obesity	Insulin resistance	Steatosis	NASH	Fibrosis	HCC
High-fat diet (HFD)	45%–75% fat, typically: 71% fat, 11% carbohydrates and 18% protein	Yes	Yes	Yes	Yes (mild)	Yes (mild)	No
High-fructose diet	73% fructose	No	Yes	Yes	Yes	Yes	No
High-fat, high-fructose diet (HFF)	HFD with high-fructose corn syrup	Yes	Yes	Yes	Yes (mild)	Yes (mild)	No
High-fat, high-cholesterol diet (HFHC)	HFD (15–45% fat) with 1% cholesterol	Yes	Yes	Yes	Yes	Yes	No
High-fat, high-fructose, high-cholesterol diet	43% fat, 17.8% high-fructose corn syrup and 2% cholesterol	Yes	Yes	Yes	Yes	Yes	No
High-fat, high glucose and fructose diet	HFD (42% fat) with 0.1% cholesterol and a high-fructose-glucose solution (23.1 g/L fructose + 18.9 g/L glucose)	Yes	Yes	Yes	Yes	Yes	Yes
High-fat, high sucrose diet	36% fat and 30% sucrose	Yes	Yes	Yes	Yes	Yes (mild)	No
Long-term low fat- high carbohydrate diet	16% protein, 73% carbohydrate, and 10.5% fat	No	?	Yes	Yes	Yes	Yes
High-fat diet + streptozotocin	HFD + 200- μ g streptozotocin injection	Yes	Yes	Yes	Yes	Yes	Yes
High-fat diet + Diethylnitrosamine (DEN)	HFD + 25 μ l/g DEN injection	Yes	Yes	Yes	Yes	Yes	Yes
High-fat diet + carbon tetrachloride (CCl ₄)	HFD + 0.08 μ l/g CCl ₄ injection	Yes	Yes	Yes	Yes	Yes	Yes
High-fat, high-fructose and high-cholesterol + CCl ₄	21.1% fat, 41% sucrose, 1.25% cholesterol and a high sugar solution (23.1 g/L fructose, 18.9 g/L glucose) + 0.2 μ l/g CCl ₄	Yes	Yes	Yes	Yes	Yes	Yes
Methionine- and choline- deficient diet (MCD)	40% sucrose and 10% fat but methionine and choline deficient	No	Yes	Yes	Yes	Yes	No
Methionine- and choline- deficient diet + DEN	MCD + 25 μ l/g DEN injection	No	Yes	Yes	Yes	Yes	Yes
Choline-deficient high-fat diet	20% protein, 35% carbohydrate, and 45% fat, without choline added	Yes	Yes	Yes	Yes	Yes	Yes
Choline-deficient amino acid diet (CDAA)	28.9 kcal/g L-glutamic acid, 15.8 kcal/g L-aspartic acid, 12.7 kcal/g L-arginine, and 10.5 kcal/g L-leucine but choline deficient	No	Yes	Yes	Yes	Yes	Yes
Choline-deficient L-amino acid-defined diet + CCl ₄	CDAA + 0.2 μ l/g CCl ₄ injection	No	Yes	Yes	Yes	Yes	Yes
<i>ob/ob</i> mice	NA	Yes	Yes	Yes	No	No	No
<i>db/db</i> mice	NA	Yes	Yes	Yes	No	No	No
<i>foz/foz</i> mice	NA	Yes	Yes	Yes	Yes	Yes	No
<i>db/db</i> mice + 25 μ l/g DEN	NA	Yes	Yes	Yes	Yes	?	Yes
Jet lag (12 h:12 h dark/light cycle disrupting every 5 days over 3 weeks by extending the dark cycle 12 h)	NA	Yes	Yes	Yes	Yes	Yes	Yes

Table 2 — Genetically modified animal models used to identify the role of JNK and p38 in NAFLD development.

MAPK	Mouse model	Phenotype	Reference
JNK1	Systemic JNK1 knockout	Under HFD: decreased body weight, increased hepatic insulin signalling, and decreased steatosis.	[46,49,52]
JNK1	Adenoviral dominant-negative JNK1 delivery to the liver	Under HFD: decreased body weight, improved insulin sensitivity, and decreased gluconeogenesis.	[50]
JNK1	Systemic antisense oligonucleotides against JNK1	Under HFD: improved insulin sensitivity and hepatic steatosis. No data on body weight.	[49]
JNK1	Liver-specific JNK1 knockdown with adenovirus	Under HFD: improved insulin sensitivity, glycolysis, triglyceride secretion, and β -oxidation.	[54]
JNK1	Liver-specific JNK1 knockout	Under CD: glucose intolerance, insulin resistance, and hepatic steatosis associated with increased gluconeogenesis and lipogenesis	[27]
JNK1	Adipose-specific JNK1 knockout	Under HFD: increased body weight and decreased insulin resistance and hepatic steatosis.	[24]
JNK2	Systemic JNK2 knockout	Under HFD: no increase in insulin sensitivity and no reduction in adipose tissue mass, but high JNK activation.	[46,49,52]
JNK2	Systemic antisense oligonucleotides against JNK1	Under HFD: improved insulin sensitivity but increased liver injury, without reducing steatosis.	[49]
JNK1/2	Systemic <i>Jnk1^{+/-} Jnk2^{-/-}</i>	Under HFD: reduced body weight and increased insulin sensitivity	[57]
JNK1/2	Liver-specific JNK1/2 knockout	Under HFD: decreased FA oxidation and ketogenesis, improving insulin sensitivity and steatosis by activation of PPAR α and FGF21 signalling.	[58]
p38 α	Liver-specific p38 α knockout	Under CD: reduced fasting glucose and impaired gluconeogenesis in an AMPK-dependent manner.	[61] [68]
p38 α	Macrophage-specific p38 α knockout	Under HFD: increased body weight, fat weight and liver weight. More glucose intolerant. Increased steatohepatitis characterised by steatosis and inflammation.	[68]
p38 γ/δ	Myeloid cells-specific p38 γ/δ knockout	Under HFHC: less steatosis-steatohepatitis and insulin resistance by M2 anti-inflammatory polarisation.	[69]
p38 δ	Myeloid cells-specific p38 δ knockout	Under HFD, HFF, and MCD: reduced neutrophil infiltration and, thus, resistant to steatosis, hepatic triglyceride accumulation, and glucose intolerance.	[69]
		Under MCD: reduced neutrophil infiltration and, thus, partially protected to steatosis.	[69]

3. STRESS KINASES IN THE CONTROL OF HEPATOCYTE METABOLISM AND STEATOSIS DEVELOPMENT

3.1. JNK

3.1.1. Activation of the hepatic JNK pathway during steatosis and NASH development

JNK is phosphorylated and activated by MKK4/7 in response to stimuli such as sugars and lipids. In animal models, JNKs are activated by hyperglycaemia inducers [28], and fructose attenuates the insulin pathway through the activation of hepatic JNK [29]. JNK is also activated in mouse liver by a high-fat diet (HFD) and genetically induced obesity [27]. These models are characterised by the enlargement of visceral adipose tissue, the secretion of free fatty acids (FFA), and the accumulation of fat in the liver, called steatosis. Moreover, hyperinsulinaemia stimulates DNL in hepatocytes [30] and, in cultured hepatocytes, these saturated FFAs activate JNK [28].

During steatosis progression, saturated FFAs activate hepatocyte lipooapoptosis in a JNK-dependent manner through Bax and the Bcl-2-interacting mediator of cell death (Bim), which triggers the mitochondrial apoptotic pathway, a crucial factor in the progression of NAFLD and NASH [31,32]. Moreover, in primary murine hepatocytes and NASH patient liver samples, the saturated FFA palmitate acts via JNK1 to increase the levels of the pro-apoptotic protein PUMA (p53 upregulated modulator of apoptosis) [33]. PUMA directly interacts with Bax and promotes caspase 3/7 activity and cell death [34]. There is also evidence that saturated FFAs activate the glycogen synthase kinase-3, promoting JNK-dependent caspase signalling that culminates in lipoapoptosis [35]. Saturated FFAs also induce hepatocyte steatosis and apoptosis by sensitising cells to TNF-related apoptosis-inducing ligand (TRAIL) and upregulating the expression of death receptor 5 (DR5) in a JNK-dependent manner [36]. Finally, saturated FFAs trigger interaction between the GTPase Cdc42/Rac1 and MLK3, leading to JNK1 activation and hepatocyte apoptosis [37]. JNK activity thus stimulates extrinsic (death receptor-mediated) and intrinsic

(organelle-initiated) apoptosis, an emergent mechanism involved in the development and progression of NAFLD and NASH [33].

Hepatic lipid accumulation and the consequent increase in fatty acid β -oxidation stimulate the mitochondrial generation of reactive oxygen species (ROS) [38], an essential element of disease progression. Oxidative stress also enhances JNK1 activity, resulting in inhibition of insulin signalling through phosphorylation of IRS-1 [39] and provoking hepatocyte death [40]. Reduced glutathione depletion, the main cellular antioxidant, also leads to JNK signalling overactivation through the stimulation of MKK4, inducing cell death in the steatotic liver [41]. Moreover, in cultured hepatocytes, overexpression of the cytochrome P450 family member CYP2E1 generates high levels of oxidants that trigger JNK activation and insulin resistance [42]. During obesity, inositol requiring (IRE) 1 α , a traducer of ER stress, leads to JNK hyperactivation and subsequent inhibition of insulin signalling [43].

3.1.2. Hepatic JNK in liver metabolism and disease

One of the main risk factors for NAFLD development is insulin resistance, and there is an inverse relationship between liver lipid content and insulin sensitivity [44]. Indeed, the first evidence implicating JNK signalling in steatosis was JNK1-mediated inhibitory phosphorylation of insulin receptor substrate 1 (IRS-1) on the serine-307, which results in insulin resistance [45]. HFD-induced obesity triggers JNK1/2 phosphorylation and activation in multiple tissues, including adipose tissue, muscle, and liver [46], and JNK1/2 activation was also observed in liver biopsies from obese patients with hepatic steatosis and NASH [47,48]. Moreover, serum glucose and insulin are reduced in HFD-fed *Jnk1^{-/-}* mice, which also show increased hepatic insulin signalling [46]. Subsequent research demonstrated that JNK1-null mice have significantly lower steatosis and liver injury than wild-type counterparts [49]. Adenoviral delivery of dominant-negative JNK1 to the liver of diabetic mice decreases gluconeogenic enzyme expression and hepatic glucose production [50]. Moreover, the administration of antisense oligonucleotides directed against *Jnk1* in HFD-fed mice was found to markedly improve insulin sensitivity and steatosis [49].

Table 3 — Genetically modified animal models to identify the role of JNK and p38 in the progression of NASH to fibrosis and, finally, HCC development.

MAPK	Mouse model	Phenotype	Reference
JNK1	Systemic JNK1 knockout	Under MCD: decreased susceptibility to NASH. Under CCl ₄ and BDL treatment: significant reduced liver fibrosis but unaltered hepatocellular injury. Under CDAA diet: significant liver fibrosis reduction. Chemically induced HCC: protection with reduced proliferation and neovascularisation.	[52,170,181,190]
JNK1	Adipose-specific JNK1 knockout	Chemically induced HCC: higher adiponectin associated with a lower incidence of HCC.	[25]
JNK2	Systemic JNK2 knockout	Under MCD: no protection against steatohepatitis. Under CCl ₄ and BDL treatment: no changes in liver fibrosis. Under CDAA diet: liver fibrosis unaltered.	[46,49,52,170]
JNK1/2	JNK1/2-specific liver knockout	Long-term JNK1/2 inhibition: altered bile acid production which leads to liver cholangiocarcinoma. Chemically induced HCC: no defects in the development of hepatitis.	[191,192]
JNK1/2	JNK1/2-specific hepatocytes and nonparenchymal cells knockout	Chemically induced HCC: protected from inflammation and tumour development.	[192]
JNK1/2	JNK1/2 deficiency in the haematopoietic compartment	Under ConA treatment (fulminant hepatitis in WT mice): profound defect in hepatitis associated with markedly reduced expression of TNF α .	[152]
p38 α	Liver-specific p38 α knockout	Under HFHC: increased severe steatohepatitis and impaired glucose intolerance. Under MCD: increased steatohepatitis with inflammatory cell infiltration, hepatic lipid peroxide and hepatic triglyceride content. Chemically induced HCC: JNK hyperactivation correlating with increased tumour burden.	[100]
p38 α	Macrophage-specific p38 α knockout	Under HFHC and MCD: less severe steatohepatitis and insulin resistance by M2 anti-inflammatory polarisation.	[100]
p38 γ	Liver-specific p38 γ knockout	Chemically induced HCC: protected against formation of liver tumours.	[199]
p38 γ/δ	Myeloid cells-specific p38 γ/δ knockout	Under HFF, MCD and jet lag: protected against steatohepatitis and fibrosis because of the reduced neutrophil infiltration.	[69,151]

Likewise, treatment with the JNK inhibitor SP600125 decreased IRS-1 phosphorylation and improved NAFLD in HFD-fed rats [51]. These data, together with the finding that *Jnk1*^{-/-} mice are less susceptible to steatohepatitis induced by a methionine- and choline-deficient diet (MCD), indicate that the activation of JNK signalling pathway occurs early after the start of the diet, when NAFLD is initiated, and parallels the development of steatohepatitis.

This JNK1 activation is critical for the development of the disease [52]. A possible explanation for the protection in JNK1 KO animals is that JNK might regulate hepatic lipid accumulation through direct effects on DNL and β -oxidation. Supporting this idea, cultured hepatocytes treated with *Jnk1*-specific antisense oligonucleotides show reduced lipogenesis and increased β -oxidation [53]. Moreover, adenovirus-driven liver-specific JNK1 knockdown was found to improve not only insulin sensitivity and glycolysis but also triglyceride secretion and β -oxidation [54]. However, because JNK1 whole-body deficiency protects against HFD-induced obesity, the improved insulin resistance and steatosis in these animals could be due to the reduction in body weight [46,50]. In line with a central role of JNK1, phosphorylation of IRS-1 on serine-307 by JNK is insufficient to explain the reduced weight gain in HFD-fed *Jnk1*^{-/-} mice, and obesity-induced insulin resistance in HFD-fed mice is blocked when IRS-1 serine-307 is replaced with the non-phosphorylatable residue alanine [55].

The specific role of JNK1 in hepatocytes remained unclear until its specific ablation in this cell type. Surprisingly, mice with hepatocyte-specific JNK1-deficiency developed glucose intolerance, insulin resistance, and hepatic steatosis on a standard chow diet (CD) [27]. The same study demonstrated that JNK1 controls the renewal of the insulin receptor in the hepatocyte cell membrane, resulting in higher insulin clearance in mice lacking hepatocyte JNK1 expression. Insulin

resistance in these mice was associated with increased gluconeogenesis and lipogenesis [27], a phenotype characteristic of type 2 diabetes [56]. These results suggest that JNK1-deficiency in other organs may compensate for the effects of hepatocyte JNK1-deficiency. Therefore, whereas other studies have suggested inhibition of JNK1 signalling as a strategy for NAFLD therapy, hepatocyte JNK1 may have hepatoprotective effects.

In contrast with the observed phenotype of JNK1-deficient animals, JNK2 knockout mice are not protected against obesity or insulin resistance [46]. Moreover, oligonucleotide-mediated JNK2 knockdown in mice was found to induce liver injury due to the elevated levels of Bim [49]. Notably, JNK2 deletion was found to result in high hepatic JNK1 activation, suggesting that some features of *Jnk2*^{-/-} mice are due to hyperactivation of JNK1 in the liver. This idea was supported because reducing JNK1 activity in *Jnk1*^{+/-}*Jnk2*^{-/-} animals protected against diet-induced obesity and insulin resistance [57]. The important role of JNK2 in liver metabolism was demonstrated by the improved insulin sensitivity and reduced HFD-induced liver steatosis after dual deletion of JNK1 and JNK2 in hepatocytes [58]. Notably, specific ablation of JNK2 in hepatocytes produced a phenotype resembling that of hepatocyte JNK1/2 dual deficiency in HFD-fed mice, confirming the crucial role of JNK2 in glycaemic regulation [58]. Mechanistically, JNK1/2 represses the nuclear hormone receptor peroxisome proliferator-activated receptor α (PPAR α) and FGF21 signalling, in part through regulating nuclear receptor corepressor 1 (NCoR1) [58]. This repression leads to an increase in fatty acid oxidation and ketogenesis that promotes the development of insulin resistance. The crucial role of FGF21 in the observed protection was demonstrated by the finding that conditional deletion of *Fgf21* and *Jnk1/2* in hepatocytes failed to protect against HFD-induced liver steatosis [59] (see Figure 1).

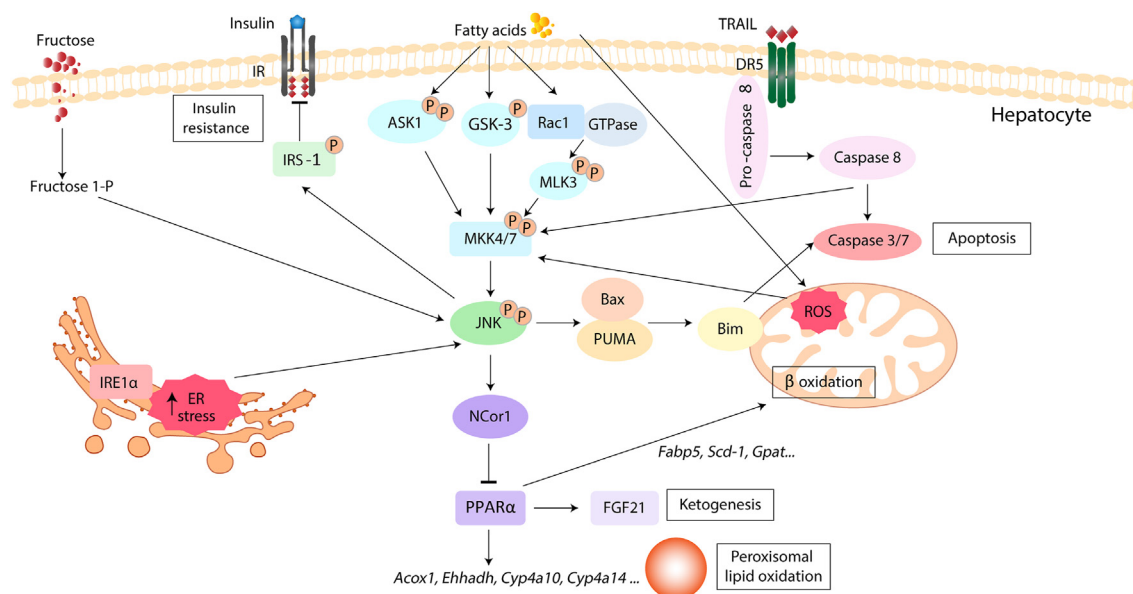


Figure 1: JNK signalling in hepatic steatosis. Increased levels of fatty acids lead to activating the JNK pathway through the phosphorylation of MKK4/7 by several kinases (ASK1, GSK-3, and MLK3). Fructose can activate JNK and induce ER stress through IRE1 α . This activation is a driver of insulin resistance by direct phosphorylation of IRS-1. JNK also promotes caspase-induced apoptosis via Bax/PUMA-Bim signalling, which can activate JNK. Finally, JNK inhibits the PPAR α pathway by activating NCor1, leading to reduced levels of β oxidation, ketogenesis, and peroxisomal lipid oxidation. The decreases in insulin sensitivity, lipid oxidation, and ketogenesis, together with the increased apoptosis, drive hepatic steatosis.

3.2. p38 MAPK

3.2.1. Hepatic p38 in steatosis development

The p38 MAPKs are in two groups, with p38 α and p38 β showing 75% amino acid sequence identity and p38 γ and p38 δ also very similar to each other (~70% identity), and the p38 γ , p38 δ pair shows more divergence from p38 α (~60% identity) [60].

p38 α has been suggested to stimulate hepatic gluconeogenesis [61]. In mice, inhibition of p38 α with pharmacological inhibitors or small interference RNA reduces hepatic glucose production by blocking the expression of key gluconeogenic enzymes such as phosphoenolpyruvate carboxykinase, glucose-6-phosphatase, and peroxisome proliferator-activated receptor γ coactivator 1 α (PGC1- α) [61]. Moreover, conditional deletion of p38 α in hepatocytes reduces fasting glucose and impaired gluconeogenesis by blocking AMPK activation after fasting [62]. p38 α is activated in the livers of obese *db/db* mice (knockout for the leptin receptor), although these mice show reduced activation of the upstream regulators MKK3 and MKK6. p38 α activation in *db/db* mice was accompanied by AMPK inhibition and hyperglycaemia, and these changes were blocked by hepatic deletion of p38 α in this mouse model [62]. The authors suggested that the inhibition of upstream regulators was mediated by the negative feedback from p38 α , whose deletion hyperactivated MKK3/6 and the protein TAK1 [63]. TAK1 hyperactivation would inhibit AMPK activation [64]. Further experiments would be necessary to define the signalling pathway controlling p38 α activation and the role of TAK1 and other p38s in this regulation.

In agreement with these results, p38 α is activated in the livers of obese mice, and expression of dominant-negative p38 α improves glucose tolerance, whereas overexpression of p38 α results in hepatic insulin resistance in *ob/ob* mice (which have a mutation in the leptin gene) [65]. These results are difficult to reconcile with a report in which feeding animals a HFD decreased p38 α / β expression [66].

Hyperactivation of p38 α / β through the expression of constitutively active MKK6 (MKK6Glu) reduced ER stress and established euglycaemia in obese and diabetic mice by enhancing nuclear translocation of Xbp1s in hepatocytes [66]. The p38 α / β inhibitor SB203580 increases the expression of lipogenic genes and triglyceride levels in both liver and isolated hepatocytes. This p38 α / β -dependent lipogenesis inhibition may be mediated by the blockade of SREBP-1c promoter activation and PGC1- β expression [67].

Finally, the recent use of the liver-specific p38 α knockout mouse model under HFD and the high-fat/high-cholesterol (HFHC) and MCD models suggest that hepatocyte p38 α protects mice from the development of steatohepatitis characterised by steatosis and inflammation [68]. In particular, mice without p38 α in hepatocytes demonstrated a decreased lipolysis and an induction of the hepatic ER stress signalling and proinflammatory cytokine production [68]. These apparently conflicting indications of hepatic p38 α / β function during obesity might reflect activation of other p38 family members recognised by the antibody and that also might be activated by the constitutive kinase MKK6. Further research is necessary to determine whether these effects are directly mediated by p38 α / β or are the result of hyperactivation of upstream kinases (and thus the modulation of other SAPKs).

This possibility is supported by the finding that p38 γ / δ are upregulated in liver biopsies from obese individuals with or without NAFLD or steatosis [69]. Both p38 γ and p38 δ are activated during liver steatosis, and whole-body deletion of these kinases protects against diet-induced steatosis in mice [69]. In line with this observation, the microbial metabolite imidazole propionate activates p38 γ , inducing the p62-mTORC1-S6K1-IRS-1 pathway, which promotes insulin resistance [70]. Imidazole propionate also induces p38 γ -mediated phosphorylation of AKT in both S473 and T308 residues in a basal condition and impairs insulin signalling [71]. Notably, basal phosphorylation of AKT without insulin stimuli in obesity has been associated with insulin

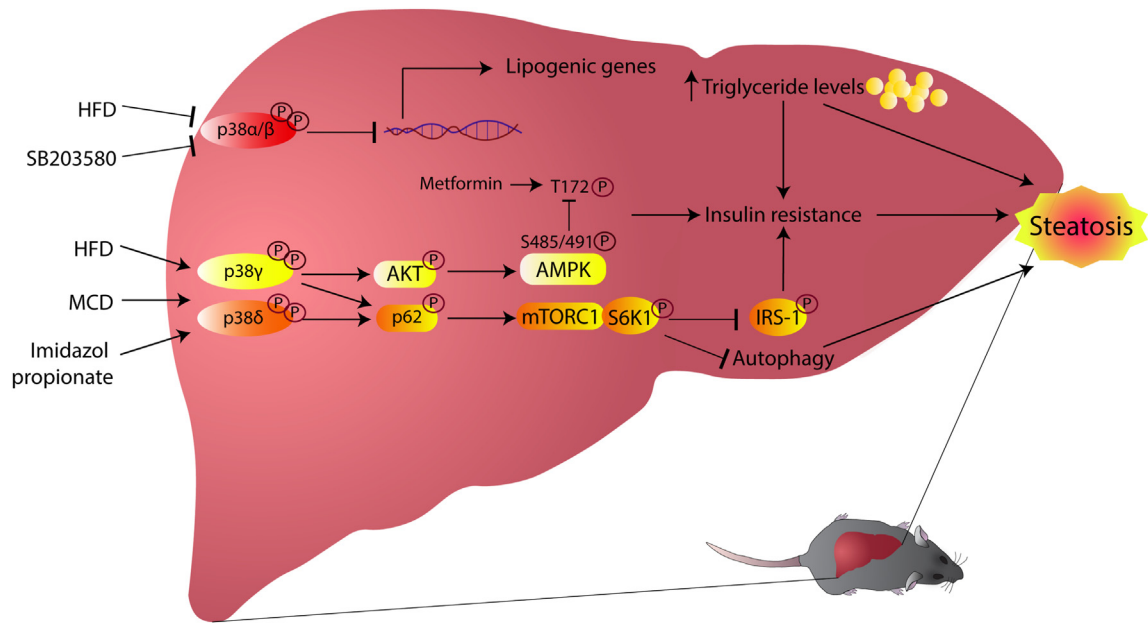


Figure 2: p38 signalling during hepatic steatosis. Hepatic p38 α/β expression decreases in livers of HFD-fed mice, leading to increased transcription of lipogenic genes, which is a driver of increased triglyceride levels. Hepatic p38 γ/δ expression is induced by HFD, MCD, and imidazole propionate. p38 γ promotes the phosphorylation of AKT, which phosphorylates AMPK on the inhibitory residues S485 and S491, driving insulin resistance. Insulin resistance is also induced by p38 γ/δ activation of p62-mTORC1-S6K1-IRS signalling, which inhibits autophagy. Insulin resistance, autophagy inhibition, and increased triglyceride levels lead to steatosis.

resistance [72,73]. This pathway induces inhibitory AMPK serine phosphorylation, subsequently inhibiting metformin-induced AMPK T172 phosphorylation [71]. Thus, imidazole propionate controls p38 γ , promoting basal AKT activation, which reduces the glucose-lowering effect of metformin [71].

Lipid metabolism might also be controlled by p38 γ/δ through the modulation of liver autophagy. Activated autophagy could attenuate liver steatosis by sequestering lipid droplets, which are eventually eliminated [74–77]. Because autophagy regulation by AMPK-mTOR is an important protective mechanism against steatosis [75,76], inhibition of this pathway by p38 γ/δ might also be an important contributor to liver steatosis [78] (see Figure 2).

3.3. SAPK upstream regulators

The kinases upstream of JNK are MKK4 and MKK7, and those upstream of p38 are MKK3 and MKK6. These kinases are activated by MAP3Ks, a family including mixed-lineage kinase, Raf kinase, apoptosis signal-regulating kinase 1 (ASK1), and MAPK kinase 1/4 (MEKK1/4) [79]. Studies have revealed the importance of these kinases in liver metabolism. For instance, the reduction in JNK activity in HFD-fed *Mlk3*^{−/−} mice attenuates liver injury by reducing hepatic steatosis and triglyceride accumulation [80]. In agreement with these data, HFD-fed *Mlk3*^{−/−} mice have low body weight, increased insulin sensitivity, and decreased macrophage recruitment linked to reduced systemic inflammation [81,82]. Not until the generation of MK2/3 double knockout mice was p38 phosphorylation analysed. The reduced JNK and p38 activation in these mice contributed to protection against HFD-induced insulin resistance and obesity [83]. The therapeutic potential of these kinases was demonstrated with the MK3 inhibitor URM099. Administration of URM099 to mice reduced JNK/p38 signalling and protected against liver injury and fibrosis without improving steatosis [83].

The MAP3K kinase ASK1 participates in NAFLD progression [84]. HFD-fed *Ask1*^{−/−} mice have reduced hepatic steatosis [85], and liver-

specific dominant-negative ASK1 transgenic mice are protected against obesity. In addition, liver-specific expression of constitutively active ASK1 increases obesity, insulin resistance, and hepatic steatosis in response to an HFD [86]. ASK1 might also be involved in the anti-steatosis effects of the secreted glycoprotein cellular repressors of E1A-stimulated genes (CREG) [87]. CREG might suppress hepatic steatosis through its direct interaction with ASK1 and the resulting inactivation of ASK1–JNK1 signalling [87]. Similar results were obtained upon deletion of the ASK1 activator hepatic Dickkopf-3 (DKK3) [86].

Recent research has demonstrated that steatohepatitis is suppressed in both mice and monkeys by CASP8 and a FADD-like apoptosis regulator (CFLAR). CFLAR disrupts the N-terminus-mediated dimerisation of ASK1, blocking the ASK1–JNK pathway without affecting p38 activation [84], and in a model of diet-induced steatosis, JNK inhibition through Silibinin-induced CFLAR activation upregulated the mRNA expression of several enzymes implicated in lipid oxidation [88]. However, although the TRAF1-ASK1-JNK/p38 signalling pathway is activated during hepatic steatosis [89], hepatic steatosis, inflammation, and fibrosis are increased in liver-specific ASK1-knockout mice [90]. Likewise, mice are protected against diet-induced hepatic steatosis and fibrosis by liver-specific ASK1 overexpression. Moreover, ASK1 expression inversely correlates with liver fat content and NASH scores in human livers [90], and phase III clinical trials of ASK1 inhibitors were discontinued due to lack of efficacy and adverse secondary effects (STELLAR 3 ClinicalTrials.gov identifier NCT03053050 and STELLAR 4 ClinicalTrials.gov identifier NCT03053063).

Liver expression of the JNK/p38 phosphatases dual-specificity phosphatase 9 (DUSP9) and MAPK phosphatase 4 (MKP4) increases insulin sensitivity by suppressing the activation of JNK and p38 [91]. Work with conditional liver-specific DUSP9-knockout mice and DUSP9-transgenic mice demonstrated that DUSP9 suppresses HFD-induced hepatic steatosis and inflammatory responses by blocking ASK1 phosphorylation and the subsequent activation of JNK and p38

signalling [92]. Likewise, HFD-fed DUSP12-deficient mice exhibit hyperinsulinaemia, insulin resistance, and liver steatosis, and hepatocyte DUSP12 overexpression ameliorates the phenotype of HFD-fed mice. DUSP12 also promotes ASK1 dephosphorylation by inhibiting JNK and p38 signalling [93]. These data consolidate the role of ASK1–JNK/p38 signalling in promoting hepatic steatosis.

However, controversy remains because DUSP14 and DUSP26 are downregulated in fatty livers, increasing the phosphorylation of JNK/p38. Liver-specific knockout of DUSP14 and DUSP26 exacerbates hepatic steatosis and increases the inflammatory response and insulin resistance in response to a HFD, and transgenic models of DUSP14 and DUSP26 expression are protected against HFD-induced effects [94,95]. Moreover, mice without DUSP10 (also called MKP5) develop insulin resistance and glucose intolerance that progresses to severe hepatic steatosis with ageing or HFD. These mice have increased p38 α / β phosphorylation in the liver, and inhibition of these kinases prevents the development of NASH by suppressing ATF2 and PPAR γ and reducing hepatic lipid accumulation, inflammation, and fibrosis [96].

MKP-1, another inactivator of both p38 and JNK, is overexpressed in liver during obesity. Work on the *mkp-1*^{−/−} mice model has demonstrated the important role of this protein in dephosphorylating JNK and p38. Surprisingly, *mkp-1*^{−/−} mice have elevated activation of these kinases but protection against steatosis and insulin resistance by increased fatty acid oxidation [97,98]. The literature has suggested that MKP-1-deficient mice are protected against hepatic steatosis due to nuclear activation of JNK/p38 and phosphorylation of PPAR α , resulting in enhanced β -oxidation [98]. Moreover, *db/db* mice without MKP-1 show suppression of PPAR γ target genes such as fat-specific protein 27 (Fsp27), a PPAR-mediated hepatic steatosis promoter [97]. Liver-specific deletion of MKP-1 enhances gluconeogenesis and hepatic insulin resistance in CD-fed mice but attenuates HFD-induced steatosis [99]. In addition, these mice have suppressed circulating levels of FGF21, suggesting that MKP-1 might be required for the expression of FGF21 in hepatocytes in a p38 α / β -dependent manner. Inhibition of p38 α / β suppressed FGF21 expression; JNK inhibition had no effect [99]. The reduced FGF21 levels in liver-specific MKP-1-deficient mice were associated with decreased skeletal muscle PGC-1 α expression, which impaired skeletal muscle mitochondrial oxidation. However, in *mkp-1*^{−/−} liver the levels of PGC-1 α were increased, resulting in increased hepatic fatty acid oxidation accompanied by reduced triacylglycerol accumulation and secretion [100]. Further research should assess whether JNK inactivation interferes with p38 α / β signalling in the regulation of liver FGF21 expression. Supporting this idea, inactivation of JNK or c-Jun suppresses increased proliferation in p38 α -deficient hepatocytes [101]. However, the relationship between these SAPKs is unknown, and the responses of p38 γ / δ isoforms have not been analysed in these upstream kinase/phosphatase models.

4. STRESS KINASES IN THE IMMUNE REGULATION OF STEATOSIS DEVELOPMENT

The liver is a metabolic organ but also contains many innate and adaptive immune cells. Among the more abundant of these cells are KCs, dendritic cells (DCs), neutrophils, and multiple types of lymphocytes. Adaptive lymphocytes include CD4⁺ and CD8⁺ T cells, as well as B cells. The liver also contains NK cells, NKT cells, mucosal-associated invariant T cells, and $\gamma\delta$ T cells [102].

During obesity, damaged hepatocytes induce immune responses by releasing saturated fatty acids and microbial derived lipopolysaccharide (LPS). These molecules are sensed in activated resident cells (e.g.,

KCs) by pattern-recognition receptors, including toll-like receptors (TLRs), and initiate proinflammatory signalling cascades within them. The JNK and p38 signalling pathways trigger the secretion of cytokines and chemokines, leading to the recruitment of monocytes, neutrophils, and various types of lymphocytes. This interferes with insulin signalling in the liver and causes systemic insulin resistance and inflammation [103–105]. Therefore, understanding the mechanisms by which immune cells are activated and recruited to the liver helps define how these cells cause injury during liver steatosis.

4.1. Innate immunity in liver steatosis

4.1.1. Macrophages and KCs

In the initial phase of NAFLD, the most important immune cell populations in the liver are the tissue resident KCs [106]. The fatty acids and LPS released by damaged hepatocytes activate KCs via the TLR4 cascade [107], inducing an M1 proinflammatory phenotype. Activated KCs then produce cytokines such as TNF- α and monocyte chemoattractant protein-1 (MCP-1). Mice without TLR4 in KCs are protected against steatosis and NAFLD progression [108]. Blocking proinflammatory M1 macrophage polarisation by depletion of p38 α prevents steatohepatitis in mice [68].

MCP1 and other cytokines and chemokines released by activated KCs promote liver infiltration by other immune cells, including monocytes and neutrophils, which contribute to hepatosteatosis development [69,109]. Therapeutic strategies to impair monocyte, macrophage, and neutrophil infiltration of the liver have succeeded in attenuating liver steatosis [69,110]. Moreover, depletion of p38 γ / δ reduces liver neutrophil infiltration and consequently protects against steatosis [69]. The importance of KCs in steatosis development has been deeply studied, and results have varied by the mouse model of NASH used. Clodronate-mediated KC depletion protects against diet-induced steatosis and insulin resistance [111,112]; the loss of KCs in diet-induced obesity is associated with an increase in hepatic steatosis and a deterioration of hepatic and systemic insulin resistance [113,114]. These discrepancies suggest that each model achieves the deletion of KCs through effects on different myeloid components. A more specific deletion of KCs would be necessary to dissect the specific contribution of these cells to liver steatohepatitis. Recently, a potent mouse model for the study of KC function was generated based on the promoter for the KC-specific gene Clec4E. Cre-specific expression was used to eliminate KCs and identify the signalling pathways involved in KC maturation from macrophage progenitors [115]. This model demonstrated that circulating monocytes infiltrate the liver and gradually adopt the transcriptional profile of the depleted KCs, assume similar functions, and become long-lived self-renewing cells [116]. These new mouse models provide useful tools for the evaluation of KC function in NASH development.

4.1.2. Neutrophils

In the context of NAFLD, neutrophils are recruited to the liver by several chemokines, including CXCR1/2 receptors and the CXCL1/2 axis (in mice) [117] and IL-8 (in humans) [118]. In addition, several studies have demonstrated that NAFLD progression requires the release of neutrophil-specific components. For instance, the inhibition of neutrophil elastase ameliorates disease severity in a mouse model of diet-induced NASH [119]. Consistent with this finding, HFD-fed mice without myeloperoxidase (MPO), a neutrophil enzyme increased in patients with NASH [120], have reduced hepatic inflammation and improved insulin resistance [121]. Moreover, neutrophil depletion protects against steatosis development by increasing lipid oxidation

and decreasing inflammation, indicating that inhibition of neutrophil trafficking is a potential treatment for steatosis [69].

4.1.3. Dendritic cells

Dendritic cells have a protective role. DCs expand and mature in NASH liver and assume an activated immune phenotype to protect it from inflammation and fibrosis [122]. DC depletion allows CD8⁺ T cell expansion and TLR expression and cytokine production in innate immune effector cells [122]. Conversely, in mice without CD103⁺ DCs, NASH progression was enhanced, and adoptive transfer of CD103⁺ DCs ameliorated liver inflammation and steatosis [123]. By contrast, CX3CR1⁺ monocyte-derived DCs secrete TNF- α during steatohepatitis progression, sustaining liver inflammation and liver injury [124].

4.1.4. NK cells

Few studies have assessed the role of NK cells in NAFLD/NASH. Patients who are obese have fewer circulating NK cells than healthy individuals, and their cytotoxic ability is lower than cells from healthy individuals [125]. By contrast, the NK cells of patients with steatohepatitis have increased levels of NKG2D and TRAIL mRNA [126]. In a recent study, DX5⁺ NKp46⁺ NK cells were found to be increased in a NASH mouse model and to inhibit the development of liver fibrosis by regulating the M1/M2 polarisation of liver macrophages [127].

4.2. Adaptive immunity in liver steatosis

The progression of NAFLD and NASH in humans and mice is characterised by an increase in the number of activated cytotoxic CD8⁺ T cells in the liver [128]. These cells are mainly recruited in response to signals mediated by IFN α [129]. The activated CD8⁺ T cells, together with NKT cells, directly activate NF- κ B signalling in hepatocytes to promote steatosis and the transition from NASH to hepatocellular carcinoma (HCC) [128]. Mice without CD8⁺ T and NKT cells are protected against steatosis and steatohepatitis [130], and the selective ablation of CD8⁺ T cells ameliorates steatohepatitis [130].

Unlike CD8⁺ T cells, CD4⁺ T cells are selectively lost because of dysregulation of lipid metabolism during NAFLD. Because CD4⁺ T cells have more mitochondrial mass, they generate higher levels of ROS than CD8⁺ T cells. Lipotoxicity during NAFLD causes oxidative damage to these cells, inducing their apoptosis and promoting liver cancer development [131]. Livers of HFD-fed mice have elevated levels of Th17 cells and IL-17 production, inducing the transition from hepatic steatosis to steatohepatitis and inflammation [132]. By contrast, HFD induces a reduction in the numbers of regulatory T (Treg) cells in liver, which protects against inflammation and NASH progression [133]. The importance of Treg cells in protecting the liver against NASH progression was further demonstrated by the finding that expanding Treg cell numbers reduce the elevated transaminase levels that appear during steatosis [134]. Mice with diet-induced NAFLD have elevated numbers of Th1 cells in liver and peripheral blood [135,136]. Additionally, liver expression of the Th1-related cytokines IFN γ , IL-12, and TNF- α increases after concanavalin A-induced hepatitis in steatotic mice fed a choline-deficient diet [137]. Patients with NAFLD have an elevated number of Th2 cells in peripheral blood and a high Th2/Treg ratio, which decreases 12 months after bariatric surgery [136]. NAFLD may also be ameliorated by the action of Th22 cells. These cells are characterised by the production of IL-22, and recombinant IL-22 has been shown to alleviate steatosis and to decrease transaminase levels [138,139].

Obese mice with mild NASH increase the apoptosis of NKT cells in the liver, concomitant with the production of Th1 cytokines [140]. In addition, adoptive transfer of NKT cells to these mice results in

decreased liver steatosis and improved glucose homeostasis [141]. NKT cells are enriched in the livers of mice with severe NASH [142]. IL-17-producing $\gamma\delta$ T cells are also increased in mice with NAFLD. Moreover, $\gamma\delta$ T cell-deficient mice have reduced levels of hepatic inflammation, transaminase, and insulin resistance [143]. HFD-fed mice also increase the production of Th1 cytokines in intrahepatic B cells. These cytokines promote Th1 cell differentiation and contribute to inflammation in NAFLD [144]. In addition, NAFLD progression promotes an increase in B cell-activating factor, considered a risk factor for NASH [145,146].

Further research is necessary to clarify the role of innate and adaptive immune cells in NAFLD/NASH. Although the understanding of the role of macrophages and other immune cells in NAFLD/NASH has recently increased, further research is necessary to investigate the relative contribution of different macrophages types (resident and circulating) and the specific role of neutrophils in the control of liver metabolism and their interaction with different liver cell types. It is also important to assess the relative contribution of the adaptive immune system to the development of this disorder.

4.3. Stress kinases in immune cells during liver steatosis

4.3.1. SAPKs in innate immunity

Given the key role of immune cells in liver steatosis and NAFLD progression and the importance of SAPKs in inflammatory processes, several studies have investigated the role of these kinases as regulators of immune cell function during NAFLD, especially in the myeloid compartment.

Macrophage deletion of p38 α impairs the innate immune response to the TLR4 ligand LPS, significantly inhibiting the production of LPS-induced cytokines by blocking the activation of the cAMP-response element-binding protein (CREB) [147]. Patients with NAFLD have high levels of p38 α in their livers, and macrophage p38 α induces lipid accumulation and proinflammatory cytokine production in hepatocytes in mice, leading to an M1 macrophage polarisation that aggravates steatohepatitis. Therefore, removing p38 α from macrophages protects against steatohepatitis [68].

Similarly, macrophage-expressed p38 γ and p38 δ control TNF- α production through the inhibition of eukaryotic elongation factor 2 (eEF2) kinase (eEF2K) [148] and the activation of ERK 1/2 [149]. eEF2K is a p38 γ/δ substrate, and p38 γ/δ deletion in the myeloid compartment protects against LPS-induced hepatitis due to reduced eEF2-mediated translation of TNF- α [148]. p38 γ and p38 δ also control the migration [150] and infiltration [69] of neutrophils to the liver. Thus, deletion of p38 γ/δ in the myeloid lineage reduces neutrophil adhesion and recruitment to damaged liver, protecting animals against diet-induced steatosis and NAFLD [69]. These results indicate that p38 γ and p38 δ in myeloid cells are potential targets for NAFLD therapy. Notably, specific deletion of p38 γ/δ in neutrophils protects mice against NASH in three dietary models: an HFD, an MCD, and a high-fat, high-fructose diet (HFF) [69]. In addition, neutrophils infiltration has been demonstrated to be crucial in controlling liver circadian rhythm, and its depletion protects against jetlag-induced steatosis [151]. In agreement with the crucial role of p38 γ/δ promoting neutrophils' infiltration in the liver deletion of these kinases, myeloid compartment also protects against jet lag-induced steatosis [151]. Thus, targeting p38 α , p38 γ , and p38 δ in the myeloid compartment may be a potent tool for impairing TLR4/LPS signalling and attenuating non-alcoholic fatty liver disease.

Mice without JNK1/2 in the haematopoietic compartment exhibit a profound defect in LPS-induced hepatitis, with markedly reduced

expression of TNF α [152]. JNK1/2 deficiency also reduces the production of inflammatory cytokines and chemokines, neutrophil/monocyte infiltration in the liver, and mortality after LPS/GalN injection, suggesting that JNK in myeloid cells promotes the development of fulminant hepatitis and regulates hepatic inflammation [153] (see Figures 3 and 4).

4.3.2. SAPKs in adaptive immunity

In contrast with myeloid SAPKs, little is known of the role of SAPKs in the lymphoid lineage during the progression of liver steatosis and NAFLD, although their function in T cell physiology has been assessed. First, JNK1/2 deficiency in the haematopoietic compartment protects against concanavalin A (ConA)-induced liver damage. This protection correlates with reduced TNF- α , suggesting an important role of JNK1/2 in TNF- α production by NKT cells [152].

The JNK pathway has been shown to play an important role in the balance between Th1 and Th2 immune responses. JNK2-deficient CD4⁺ T cells exhibit a defect in IFN- γ production during the early stages of differentiation. Consequently, CD4⁺ T cells differentiate poorly into effector Th1 cells but normally into Th2 cells [154].

JNK1 is also required for CD8⁺ T cell expansion and activation *in vitro*. JNK1 deficiency in CD8⁺ T cells results in reduced IL-2 and IFN- γ production. Moreover, JNK1 mediates the transcription of AP-1 in CD8⁺ T cells [155]. Because the impairment of CD8⁺ T cell expansion attenuates liver steatosis development, further research in mouse

models with JNK1 depletion in CD8⁺ T cells might elucidate the role of JNK1 in the setting of NAFLD.

Th1/Th2 balance is also regulated by p38 α , and p38 α deficient CD4⁺ T cells preferentially differentiate into Th2 phenotype because of increased endogenous production of IL-4 [156]. The lack of p38 α inhibits AKT and enhances ERK activation, which might result in decreased Th1 and increased Th2 differentiation [157,158]. In the setting of NAFLD, the absence of p38 α in CD4⁺ T cells may lead to attenuation of the disease by promoting Th2 differentiation and decreasing Th1 cells in the liver. Furthermore, the lack of p38 α in Th1 cells impairs their ability to secrete large amounts of IFN- γ in response to IL-12 and IL-18 [159]. IFN- γ secreted by Th1 cells is implicated in M1 macrophage polarisation, promoting NAFLD progression; therefore, mice conditionally lacking p38 α in T cells might be a potent model for studying the role of p38 α in liver steatosis.

Activation of p38 α signalling in CD4⁺ T cells plays a pivotal role Th17 cell function by regulating IL-17 production at the translational level through indirect activation of eIF-4E (eukaryotic translation initiation factor 4E) by MAPK-interacting kinase (MNK), a p38 α target. p38 α contributes to Th17 through an alternative activation pathway involving Zap70-mediated phosphorylation of p38 α on Tyr323 [160]. Because Th17 cell-derived IL-17 participates in NAFLD progression and mice lacking an IL-17A or IL-17A receptor have less steatosis [161,162], research on the relative contribution of p38 α in this process would contribute to the literature.

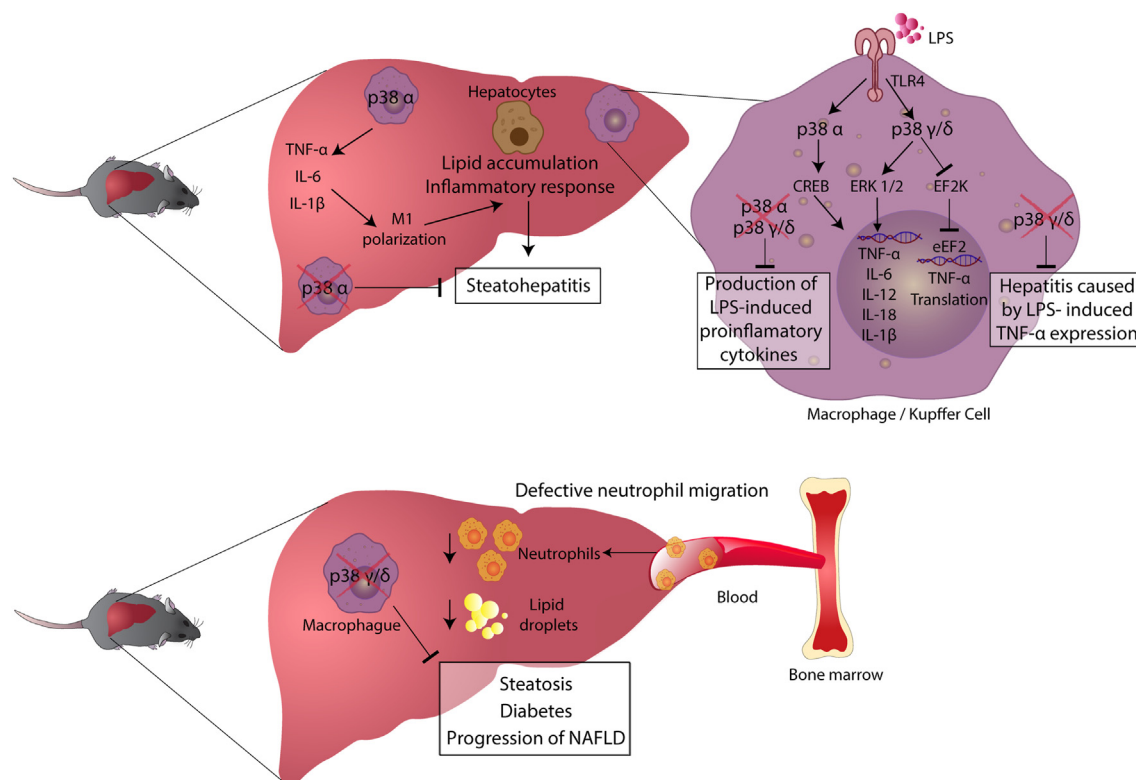


Figure 3: Role of myeloid p38 during liver steatosis and NAFLD. Macrophage p38 α promotes the progression of steatohepatitis by inducing cytokine production and M1 polarisation, leading to lipid accumulation in hepatocytes and potentiating the inflammatory response within them. Myeloid p38 α is also implicated in the LPS response in macrophages through the activation of cAMP-response element-binding protein (CREB), leading to the production of proinflammatory cytokines and chemokines. Myeloid p38 γ and p38 δ are also involved in the production of cytokines in response to LPS and control TNF- α expression through the activation of ERK 1/2 or through the phosphorylation and inactivation of eEF2K. Once eEF2K is inactivated, eEF2 is dephosphorylated and activated, allowing the translational elongation of nascent TNF- α and promoting hepatitis development. Myeloid p38 γ and p38 δ also control neutrophil migration to damaged liver: lack of p38 γ/δ in the myeloid compartment results in defective neutrophil migration; decreased hepatocyte lipid accumulation; and protection against steatosis, diabetes, and NAFLD progression.

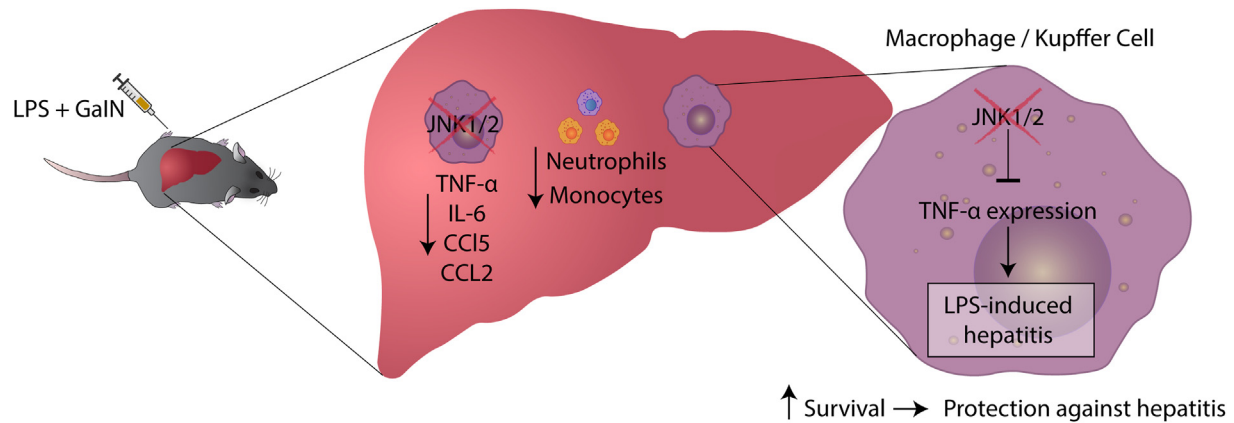


Figure 4: Role of myeloid JNK during liver steatosis and NAFLD. Lack of JNK1/2 in the myeloid compartment leads to the suppression of hepatitis and increased survival in a model of acute liver injury induced by LPS + GalN, featuring markedly reduced expression of proinflammatory cytokines (TNF- α , IL-6) and chemokines (CCL5, CCL2) and reduced liver infiltration by monocytes/neutrophils.

Additionally, mice lacking both p38 α and β in naïve CD4⁺ T cells show enhanced differentiation into Treg cells due to reduced mTOR activation [163]. Moreover, genetic ablation of p38 α in T and NKT cells protects mice from liver inflammation by reducing the production of TNF α and IFN- γ [164]. Thus, studying whether liver steatosis progression is attenuated by p38 α deletion in T and NKT cells in mouse models of NAFLD/NASH would contribute to the literature.

SAPKs play an important role in T cell function; however, how the expression and activation of these kinases in T cells affects liver metabolism and steatohepatitis development remains unclear. To evaluate their function, more studies are necessary in mice with T cell-specific deficiency for these kinases in mouse models of diet-induced NAFLD/NASH.

5. STRESS KINASES CONTROL OF FIBROSIS DEVELOPMENT

Non-alcoholic steatohepatitis (NASH) is a progressive form of NAFLD in which, in addition to fat accumulation in the liver, there is increased inflammation and hepatocyte damage. This hepatocellular injury causes hepatic fibrosis, the strongest predictive factor for liver-caused mortality, because of its evolution to cirrhosis (fibrotic scarring) and HCC [165]. The main effectors of fibrosis are the hepatic stellate cells (HSCs) and their derived cells, the myofibroblasts [166]. During liver injuries, JNK plays an important role in both HSCs [167,168] and myofibroblasts [169] where JNK is activated in fibrotic livers from mice and patients [170]. Activation of HSCs during hepatic fibrogenesis is characterised by expression of α SMA and their proliferation and migration to the necrotic area, where they synthesise extracellular matrix proteins to repair the damage [171]. There is a strong activation of JNK in fibrotic NASH livers [170], and activation of the JNK-p70S6K pathway in HSCs preceded the transformation into myofibroblasts (detected by α SMA expression) [171].

In HSCs, transforming growth factor β (TGF β) and platelet-derived growth factor (PDGF) induce JNK activation and the phosphorylation of Smad2/3 after liver injury in both murine and patients NASH livers. JNK participates in the development of liver fibrosis induced by bile duct ligation (BDL) and chemical induction with carbon tetrachloride (CCl₄). Mechanistically, JNK participates in the HSC migration as JNK inhibitor SP600125 inhibited TGF β and PDGF-induced migration of resident HSCs. Recent publications have also suggested that TGF- β 1-induced autophagy is involved in the activation of hepatic stellate cell

through activation of the ERK and JNK [172]. Notably, CCl₄-induced liver inflammation, necrosis, and fibrosis are prevented by naringenin inhibition of TGF β -JNK-Smad3 pathways [173]. Additionally, the miR-6133-5p has antifibrotic effects because of the inactivation of TGF β R2 and JNK [174]. Recently, the Fst1 neutralising antibody (22B6 mAb) was demonstrated to downregulate JNK phosphorylation and TGF- β 1 induced phosphorylation of Smad2 attenuating the CCl₄-induced liver fibrosis [175]. JNK also contributes to α -smooth muscle actin (α SMA) expression in HSC activation and migration, co-localising in fibrotic areas in mice and livers from patients with NASH [170]. In addition, angiotensin II (AngII), another profibrogenic mediator, activates JNK [176], and its inhibition reduces experimental fibrogenesis in mice [170]. All these data support the role of JNK in the development of liver fibrosis.

Recently, a new model of steatohepatitis-associated fibrosis has been studied. Concretely, HFD induces liver fibrosis in mice without CYP2A5 (antioxidant induced by CYP2E1) and PPAR α . In Ppar α -/-/Cyp2a5-/- mice there is increased ROS production, phosphorylation of JNK, and formation of nitrotyrosine adduct while thioredoxin and glutathione is suppressed. The inflammation and fibrosis in this model were observed within the clusters of lipid droplets co-localising with CYP2E1 in hepatocytes [177]. Hepatocytes induce HSC activation by the generation of ROS promoting lipid peroxidation and steatofibrosis. This finding is not surprising because JNK1/2 represses PPAR α by NCoR1 [58] and JNK regulates CYP2E1-mediated oxidative stress [42]. The role of ROS and JNK in fibrosis was also corroborated in a human stellate cell line (LX-2 cells) where decreased β -arrestin2 protects against liver fibrosis by downregulating ROS production through the JNK-NOX4 pathway [178].

Notably, JNK1-deficient mice presented a significant reduction of CCl₄ and BDL-induced fibrosis, whereas fibrosis remained unchanged in JNK2-deficient mice [170]. Therefore, JNK1 but not JNK2 is suggested to promote fibrogenesis. However, the hepatocellular injury remains unaltered when using pharmacologic JNK inhibition or genetic JNK1 deficiency. Data using the JNK inhibitor SP600125 had reached the same conclusions using different models of liver injury [179]. Remarkably, JNK is not activated in the KCs under this liver injury and macrophage infiltration was not reduced in SP600125-treated mice, suggesting that inflammation is not the JNK-induced fibrosis mechanism [170]. Thus, the reduced fibrogenesis was not secondary to reduced injury.

Other studies have attempted liver fibrosis using dietary models of NASH-induced hepatic fibrosis. As aforementioned, systemic JNK1 knockout demonstrated a decreased susceptibility to NASH under MCD, whereas JNK2 null mice demonstrated no protection against steatohepatitis using the same diet [52]. Considering that the MCD diet results in minimal fibrosis with substantial body weight loss, a choline-deficient L-amino acid-defined (CDAA) diet model was used to mimic human NASH-fibrosis-HCC in rats and mice [180,181]. The results demonstrated that JNK1 null mice presented a significant liver fibrosis reduction, whereas in JNK2 null mice fibrosis was unaltered. Notably, by using bone marrow transplantation, JNK1, but not JNK2, in KCs was demonstrated to promote the progression NAFLD to NASH and, finally, fibrosis by inducing a chronic inflammation [181]. These data contrast with the chemically or physically induced fibrosis results, proving that JNK1 may promote fibrosis by using different mechanisms. In conclusion, JNK1 is an important mediator of the fibrogenic response by participating in the TGF β and AngII pathways, by increasing the ROS production, and by mediating the chronic inflammation.

The importance of the upstream JNK kinases such as ASK1 in fibrosis has also been analysed. ASK1 is activated in oxidative stress leading to hepatocyte apoptosis, liver inflammation, and fibrosis through JNK and p38 signals. Selonsertib is a selective inhibitor of ASK1, and its use suppressed the growth and proliferation of HSCs by inhibiting p38 and JNK, alleviating fibrosis in rats [182]. The same finding was reported by using the inhibitor GS-444217 [183]. However, as aforementioned, the phase III clinical trials with this inhibitor were discontinued. Finally, DUSP10/MKP5-deficient mice that presented an increased p38 α / β phosphorylation in the liver also demonstrated reduced liver fibrosis [96].

6. STRESS KINASES IN HCC

The high prevalence of HCC has long been related to hepatitis B and C; however, the increasing incidence of HCC might be because of the increase in NASH due to the obesity epidemic [184]. SAPKs are multitasking kinases involved in the mechanisms that control the progression of obesity to NASH and HCC and in all aspects of cancer biology, including inflammation, proliferation, cell survival, differentiation, migration, and metastasis [185]. The pro- tumourigenic or anti- tumourigenic actions of SAPKs depend not only on the family member but also on the type of cancer.

Hepatocytes require JNK to proliferate, and JNK1 (not JNK2) is activated in human primary hepatocellular carcinomas [186]. A study of 31 primary human HCCs found that JNK1 was highly activated in 55% of them. This increase in JNK1 activation is associated with larger tumours and altered histone H3 methylation, resulting in elevated expression of cell growth genes [187]. JNK activation in human biopsies was corroborated by the activation of its substrate c-Jun [188]. This transcription factor antagonises the proapoptotic function of p53, and its inactivation reduces tumour formation [189]. In line with these findings, mice lacking JNK1 are protected against chemically induced HCC, showing reduced tumour proliferation and neovascularisation [190]. These data suggest that pharmacological inhibition of JNKs would be a viable strategy for the treatment of HCC. However, recent studies have shown that the absence of JNK1/2 in hepatocytes alters bile acid production, leading to liver cholangiocarcinoma [191]. Care is therefore warranted with the long-term use of JNK inhibitors.

In addition to its function in the liver, JNK facilitates HCC progression by increasing the liver expression of the inflammatory cytokines TNF α and IL-6 [152]. Inflammation and tumour development are prevented in

mice lacking JNK1 and JNK2 in hepatocytes and nonparenchymal cells [192]. JNK appears to function in hepatocytes to reduce tumour development [192], and mice lacking JNK1/2 in hepatocytes develop cholangiocarcinoma. This finding suggests that the protective effect of JNK1/2 deletion is due to the absence of these kinases in the myeloid compartment [153]. In addition of the role of JNK1 in myeloid and liver cells in HCC development, recently, mice lacking JNK1 in adipose tissue have been demonstrated to be protected against chemical-induced HCC [25]. Notably, activation of JNK is higher in male adipose tissue correlating with the greater HCC incidence [25]. The authors demonstrated that adipose JNK1 controls adiponectin levels affecting HCC development [25].

Hepatic p38 α was first described as a suppressor of the oncogenic effects of H-Ras [193] and protects against chemically induced HCC in mice, probably due to its inhibitory effect on JNK activation. Moreover, mice lacking p38 α in hepatocytes have JNK hyperactivation, correlating with an increased tumour burden after chemically induced HCC [101]. Whereas JNK1 activation promotes ROS accumulation and cell death in hepatocytes, p38 α suppresses both liver damage and carcinogenesis stimulated by the release of IL-1 α [194]. Notably, the higher activation of liver p38 α in women than in men, due to higher circulating levels of the hormone adiponectin, partly explains the higher prevalence of HCC in men [25].

The outcome of blocking a signalling pathway depends substantially on the context, for example, p38 α inhibition improves the efficacy of sorafenib, the only systemic treatment approved for advanced HCC. This multikinase inhibitor (which increases patient survival by 2.8 months [195]) activates p38 α and, thus, stimulates the ERK and ATF2 signalling pathways, involved in tumour resistance to sorafenib [196]. A study of the livers of 20 patients with HCC found lower activity of p38 and its upstream kinase MKK6 in the tumour than in the surrounding healthy tissue [197]. Although the authors could not identify the p38 family member(s) involved, the relative abundance of the different members, together with the ability of the inhibitor SB203580 to prevent MKK6-induced apoptosis in hepatoma cell lines, makes p38 α the most likely candidate. The anti-tumourigenic effects described for p38 α partly rely on the phosphorylation of the N-terminal domain of retinoblastoma tumour suppressor protein (Rb), which blocks Rb inactivation by cyclin-dependent kinases, delaying cell cycle progression [198]. Rb is also phosphorylated by p38 γ , but in different domains and with opposite effects; p38 γ inactivates Rb, initiates cell cycle entry after injury, and induces cell proliferation [199]. These mechanistic data are relevant because human HCC biopsies have higher levels of p38 γ than control biopsies do. In mice, both the absence of p38 γ and its inhibition by pirfenidone protect against chemically induced HCC [199]. The correlation of low the expression of p38 γ [199] and p38 δ [200,201] with survival in human HCC illustrates the necessity for specific inhibitors of the individual p38 family members to define their role in cancer progression and to develop novel cancer treatments (see Figure 5).

7. SAPK INHIBITORS FOR LIVER DISEASE THERAPY

Chronic activation of SAPKs ultimately causes metabolic changes associated with obesity and its related diseases, and SAPKs become potential targets in the context of metabolic syndrome. Therapeutic strategies to treat obesity and metabolic diseases using SAPKs as targets are mainly focused on the development of inhibitors. There have not been SAPK inhibitors in clinical trials for the treatment of NAFLD, NASH, and HCC, but several studies have indicated that the inhibition of SAPK pathways would protect against these diseases.

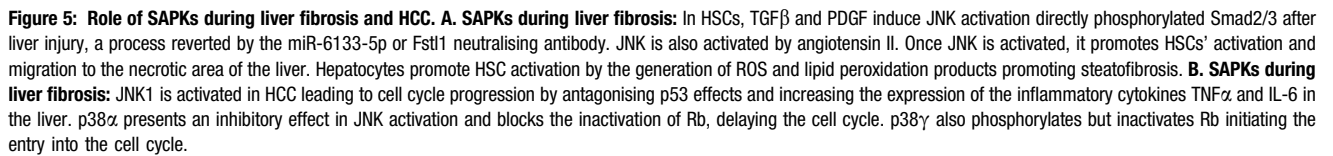


Figure 5: Role of SAPKs during liver fibrosis and HCC. A. SAPKs during liver fibrosis: In HSCs, TGF β and PDGF induce JNK activation directly phosphorylated Smad2/3 after liver injury, a process reverted by the miR-6133-5p or Fstl1 neutralising antibody. JNK is also activated by angiotensin II. Once JNK is activated, it promotes HSCs' activation and migration to the necrotic area of the liver. Hepatocytes promote HSC activation by the generation of ROS and lipid peroxidation products promoting steatofibrosis. **B. SAPKs during liver fibrosis:** JNK1 is activated in HCC leading to cell cycle progression by antagonising p53 effects and increasing the expression of the inflammatory cytokines TNF α and IL-6 in the liver. p38 α presents an inhibitory effect in JNK activation and blocks the inactivation of Rb, delaying the cell cycle. p38 γ also phosphorylates but inactivates Rb initiating the entry into the cell cycle.

The main problem of this inhibitor is its toxicity and reduced specificity because ATP-competitive inhibitors would indiscriminately inhibit the phosphorylation of all JNK substrates and also might affect other kinases [205–207].

Moreover, JNK-interacting protein-1 (JIP1) is a scaffolding protein that enhances JNK signalling by creating a proximity effect between JNK and upstream kinases. Small molecules that block JNK-JIP1 interaction act as competitive inhibitors of JNK. BI-78D3 inhibits the phosphorylation of JNK substrates both *in vitro* and in cell culture. Additionally, in animal studies, BI-78D3 not only blocks JNK-dependent Con A-induced liver damage but also restores insulin sensitivity in mouse models of type 2 diabetes [203].

Finally, JNK inhibitors have not been developed to treat patients with HCC, but JNK's roles in hepatocyte death and compensatory proliferation make them promising anti-HCC therapies. An inhibitory peptide directed against the substrate-docking domain of JNK proteins (D-JNK1) suppressed JNK activity and reduced tumour growth in the DEN-induced HCC model and in a human HCC xenograft model [186]. In a rat DEN-induced HCC model, the administration of the JNK inhibitor SP600125 reduced the number and size of HCCs [208], and JNK inhibition by SP600129 enhances apoptosis and reduces human HCC cell growth induced by the tumour suppressor WWOX [209]. Additionally, inhibition of JNK has been shown to improve the efficacy of some current chemotherapeutic agents. For example, SP600125, in combination with the chemotherapy drug TNF-related apoptosis-inducing ligand (TRAIL), was shown to increase apoptosis in human HCC cultures [210]. JNK1 correlates directly with poor therapeutic response to sorafenib, a multikinase inhibitor that is the only treatment approved for HCC [211]. However, long-term JNK inhibition alters

cholesterol metabolism and bile acid homeostasis, increasing intrahepatic cholangiocarcinoma [191]. Therefore, when deciding to use JNK inhibitors for the treatment of steatosis, the risk of cholangiocarcinoma development should be considered.

Regarding to p38 MAPKs inhibitors, although many pharmaceutical companies have ongoing clinical trials, some have failed due to safety issues. There are p38 MAPK inhibitors that have been progressed in clinical trials related to inflammatory diseases including rheumatoid arthritis, Alzheimer's disease, and inflammatory bowel disease [212]. However, the effect of p38 inhibitors in the treatment for liver inflammatory diseases including NAFLD, NASH, and HCC remains unknown. SB203580 inhibitors have been demonstrated to block the production of proinflammatory cytokines such as TNF- α and IL-1 β in inflammatory disease models [213–215]. SB203580 administration to mice, which inhibits p38 α , was reported to prevent steatohepatitis induced by an HFHC diet. After SB203580 treatment, glucose intolerance was improved, liver inflammation and lipid accumulation in hepatocytes were decreased, expression levels of TNF- α and IL-6 were also reduced, and M2 anti-inflammatory macrophage polarisation was restored [103]. The same results were observed after the treatment with BIRB796 inhibitor [103]. However, another study demonstrated that chemical inhibition of p38 α/β using SB203580 increased the expression of lipogenic genes in the liver from fasted animals and elevated triglyceride accumulation [62]. Additionally, LY-2228820 and PH-797804, which are p38 α/β inhibitors, markedly attenuated hepatocyte death and reduced oxidative stress, neutrophil infiltration, inflammation, and fibrosis in a HFD-induced NASH model [216]. Pirfenidone, a p38 γ inhibitor, also exerts a protective effect against DEN-induced HCC [199]. In addition, markedly attenuates liver fibrosis in the rodent model of human NASH with a significant reduction of hepatocyte apoptosis and hepatic crown-like structures formation, reducing the expression of genes related to lipogenesis and fatty acid synthesis and enhancing the expression of those related to fatty acid oxidation. It also reduces insulin resistance, hepatic inflammation, and fibrosis in mice with pre-existing NASH [217,218]. Moreover, sorafenib leads to the p38 α -dependent activation of ERK and ATF2 signalling that finally results in a poor response to sorafenib therapy in human HCC. A combination of sorafenib and p38 α inhibition might be a promising approach to overcoming therapy resistance of human HCC, because pharmacological silencing of p38 α was found to sensitise mouse HCC to sorafenib treatment and prolong survival [192]. Finally, inhibition of p38 γ by pirfenidone also protects mice against the chemically induced formation of HCC [195].

In conclusion, although many studies on SAPK inhibitors have been conducted, no drug has been developed for clinical use, of their non-specificity and side effects. Thus, further investigation is necessary in this field to develop selective inhibitors for the target tissue and each JNK or p38 family member. Notably, one problem with the therapeutic use of JNK inhibitors is that they do not target a specific JNK isoform and that each of them might have a specific function dependent on the tissue and even opposite effects in metabolism. JNK inhibitors have been used clinically without considering the context of tissue-dependent expression and role of the JNKs, or the pathological significance of each isoform, leading to the lack of efficacy and the side effects observed in clinical trials. In the case of p38 MAPKs inhibitors, they could specifically inhibit p38 activity in hepatic nonparenchymal cells including macrophages, leading to the amelioration of experimental steatohepatitis, providing a potential rationale for clinical trials to investigate p38 inhibitors for the treatment of NAFLD and NASH. However, selective inhibitors for p38 α and p38 β seem to produce p38 γ and p38 δ hyperactivation [219], due to the negative feedback

that p38 α has over the upstream activators of the pathway, MKK3/6, which might lead to harmful side effects. Therefore, more studies with animal models in which different isoforms of p38 are depleted in different tissues are required. Finally, a deeper investigation of the networking and scaffold interacting proteins every isoform in the different tissues is essential to further understand the organisation of SAPK pathway and to provide potential therapeutic strategies for the prevention and treatment of metabolic syndrome and associated disorders.

8. PERSPECTIVES

In this review, we highlighted the important role of SAPKs in steatosis and its progression to HCC. However, the specific role of each isoform in steatosis development remains unclear, mainly because of the interactions and compensatory effects between family members. Moreover, the cell-specific effects are unknown for many isoforms. This absence of information is particularly the case with the p38 family, because until recently, most research has focused on p38 α , with little attention paid to the important effect of this kinase on the activation of the other family members. Thus, some described p38 α functions might be due to the activation of other p38 kinases. In addition, most research in this area has been performed with pharmacological inhibitors or whole-body deletion models. Likewise, although many SAPK substrates are known, uncertainty remains about the substrates involved in liver steatosis. Clarification of the complex mechanisms by which SAPKs control liver metabolism and the development of liver steatosis requires models of each SAPK isoform in specific cell types. For instance, the development of mice with JNK2-deficiency in CD4⁺ T cells would provide an alternative model for studying the role of SAPKs in liver steatosis and NAFLD.

This review focused on the liver and the immune system; however, liver metabolism is also regulated by the activation of SAPK in adipose tissue and skeletal muscle [26] and the effects of these kinases in the central nervous system (CNS) [23]. Moreover, the tissue-specific effects of SAPKs demonstrate their tight regulation and the differences in substrate selectivity. Deciphering the interactions between SAPK isoforms, their regulation, and their cell type-specific substrates holds promise for defining their contribution to hepatic steatosis and HCC and, in the long term, developing effective treatments. The implication of SAPKs in the regulation of liver metabolism and the development of obesity has been demonstrated, and the potential of these kinases as targets for the treatment of liver metabolic conditions has been evaluated. However, the molecular mechanisms that control the *in vivo* effects underlying these strategies are not fully understood, and further research is necessary before clinical applications can be envisioned.

AUTHOR CONTRIBUTION

All authors were responsible for drafting the article and revising it critically for important intellectual content. All authors approved the publication of the final version of the manuscript.

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CONFLICT OF INTEREST

None declared.

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