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Review

Advancing the understanding of NAFLD to hepatocellular carcinoma development: From experimental models to humans

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ABSTRACT

Nonalcoholic fatty liver disease (NAFLD) has recently been recognized as an important etiology contributing to the increased incidence of hepatocellular carcinoma (HCC). NAFLD, characterized by fat accumulation in the liver, is affecting at least one-third of the global population. The more aggressive form, nonalcoholic steatohepatitis (NASH), is characterized by hepatocyte necrosis and inflammation. The development of effective approaches for disease prevention and/or treatment heavily relies on deep understanding of the mechanisms underlying NAFLD to HCC development. However, this has been largely hampered by the lack of robust experimental models that recapitulate the full disease spectrum. This review will comprehensively describe the current *in vitro* and mouse models for studying NAFLD/NASH/HCC, and further emphasize their applications and possible future improvement for better understanding the molecular mechanisms involved in the cascade of NAFLD to HCC progression.

1. Introduction

Hepatocellular carcinoma (HCC), a major type of primary liver cancer, is the second leading cause of cancer mortality globally. The classical risk factors of HCC include viral infections (HBV and HCV), aflatoxin B1, tobacco exposure and alcohol consumption. Recently, nonalcoholic fatty liver disease (NAFLD) has been recognized as an emerging risk for developing HCC [1,2]. NAFLD represents the most frequent chronic liver disease with the prevalence of 25–30% worldwide. A percentage varying between 4% to 22% of HCC cases has been estimatedto be ascribed to NAFLD [3].

NAFLD is defined by pathologic accumulation of fat in the liver without experience of alcohol abusing. Thus, hepatic steatosis is the hallmark of NAFLD, which is histologically diagnosed when fat accumulation occurs in 5% of hepatocytes [4]. According to the percentage of steatotic hepatocytes, NAFLD is graded into mild (0–33%), moderate (33–66%) and severe (> 66%) [5]. It progresses from simple liver steatosis (NAFLD) to nonalcoholic steatohepatitis (NASH), and in more severe cases, to liver fibrosis and cirrhosis [6]. NASH with fibrosis or cirrhosis increases the risk of developing HCC [7]. The most common causes of NAFLD are obesity, insulin resistance and metabolic syndrome [8]. However, the exact pathogenetic mechanisms responsible for NAFLD to HCC progression remain largely unclear.

Knowledge originated from clinical or population studies are mainly descriptive. Thus, in-depth mechanistic understanding and future therapeutic development require robust experimental models. In this study, we aim to overview the state-of-the-art of the current *in vitro* and mouse models for studying NAFLD/NASH/HCC, and emphasize their

Abbreviations: AMPK, AMP-activated protein kinase; BMI, body mass index; BMAL1, brain and muscle-ANRT-like protein 1; CCl4, carbon tetrachloride; CLOCK, circadian locomoter output cycles protein kaput-like; db/db, leptin resistant; DEN, diethylnitrosamine; ESC, embryonic stem cells; FFAs, free fatty acids; GWAS, genome-wide association studies; HCC, hepatocellular carcinoma; HF, high-fructose; HFD, high-fat diet; HGF, hepatocyte growth factor; HLC, hepatocyte-like cells; JAK/STAT, Janus kinase/signal transducer and activator of transcription; JNK, c-Jun N-terminal kinase; KCs, kupffer cells; LEPRb, leptin receptor type B; LPS, lipopolysaccharide; MCDD, methionine-choline deficient diet; Mc4r, melanocortin 4 receptor gene; NAFLD, nonalcoholic fatty liver disease; NLRPs, nod-like receptor proteins; NASH, nonalcoholic steatohepatitis; ob/ob, leptin-deficient; PTEN, hepatocyte-specific phosphatase and tensin homolog; PNPLA3, patatin-like phospholipase domain-containing 3; PPARs, peroxisome proliferator activated receptors; PPARα, peroxisome proliferator activated receptor-α; RORγt, orphan nuclear receptor; ROS, reactive oxygen species; TLR4, toll-like receptor-4; Th17 cell, T helper 17 cell; TGF-β, transforming growth factor-β; TNF-α, tumor necrosis factor-α; TRAIL, TNF-related apoptosis-inducing ligand; VLDL, very low-density lipoprotein

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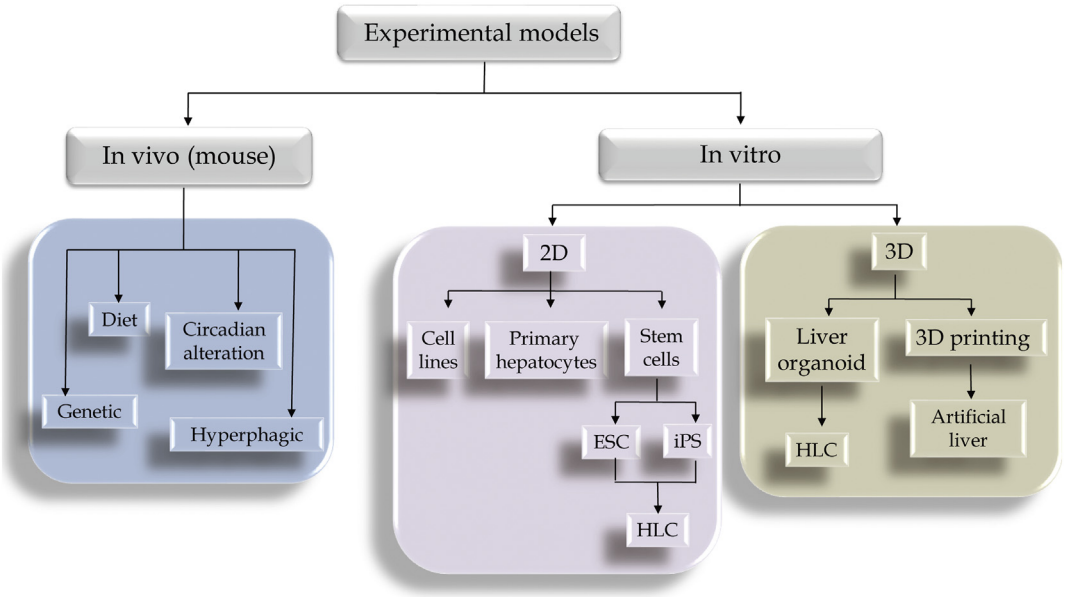

Fig. 1. Summary of experimental models applied in NAFLD/NASH/HCC research. ESC: embryonic stem cells; iPS: induced pluripotent stem cells; HLC: hepatocyte-like cells.

applications and possible future improvement for better understanding the molecular mechanisms involved in NAFLD to HCC development.

2. Exploration of experimental models for studying NAFLD/NASH/HCC

Robust experimental models are essential for determining the mechanisms of pathogenesis and developing therapeutic strategies for NAFLD/NASH/HCC. *In vitro* models would advance mechanistic studies and therapeutic development. Animal models can mirror both the histopathology and pathophysiology of each stage and the progression of the disease. This part will focus on the development of *in vitro* and mouse models (Fig.1 and Table 1).

2.1. 2D and 3D in vitro models

The general principle of the *in vitro* models is based on the exposure to saturated or unsaturated long chain fatty acids resulting in steatosis [9,10]. In this respect, HCC cell lines have been widely used; whereas these cells are far from recapitulating the physiology of primary hepatocytes. Isolated primary hepatocytes have been explored to model this disease [11], but these cells are technically challenge to be maintained in cell culture. Cultivation of primary human hepatocytes in 3D spheroid has recently been explored for modeling steatosis and insulin resistance [12]. Sophisticated approaches have been developed for generation of hepatocyte-like cells (HLC) from pluripotent stem cells including embryonic stem cells (ESC) and induced pluripotent stem cells [13]. Induction of a NAFLD-like phenotype has been observed by exposuring ESC-derived HLC to a nutrient cocktail of lactate, pyruvate and octanoic acid [14].

Organoids are “mini-organs” on a dish in 3D culture that are generated from adult stem cells. Compared to 2D culture of immortalized cell lines, they are much better in recapitulating the architecture, composition, diversity and organization of the original organ/tissue [15]. These systems are currently being widely explored for stem cell research and disease modeling [16]. The liver harbors resident stem cell populations that can be cultured into organoids *in vitro* [17]. These liver organoids can be maintained and scaled up in an expansion medium for long-term culture or differentiated into HLC with a defined lineage differentiation medium. A recent study has demonstrated that liver organoids from mouse, cat, dog or human can accumulate lipids upon feeding with fatty acids [18]. This has provided proof-of-concept of modeling steatosis using liver organoids, encouraging researchers to further improving this model system.

3D bio-printing with human cells and hydrogel scaffolds has led to fabrication of liver tissue-like constructs or artificial livers [19]. With the advance of stem cell and organoid technologies, scalable production of stem cell-derived HLC or liver organoid cells has greatly harnessed 3D liver printing [20]. Currently, it remains a technical challenge to construct blood vessel-like channels to supply oxygen and nutrients to hepatocytes [21]. In respect to generation of NAFLD models, although no report yet, we propose two scenarios: exposure of cells to fatty acids pre- or post-printing. However, the second approach requires well-established vessel channels in the printed liver.

2.2. Dietary mouse models

Dietary NAFLD models mainly include high-fat diet (HFD), methionine-choline deficient diet (MCDD) and high-fructose (HF) diet. All of them trigger NAFLD/NASH development and progression. Classic HFD mouse models fed a diet composed of 71% fat, 11% carbohydrates and 18% protein [22]. HFD leads to hepatic steatosis and elevated serum insulin levels, but absent of liver inflammation [23].

MCDD consists of substantial sucrose (40%) and moderate amount of fat (10%), but is deficient in methionine and choline. It reduces VLDL synthesis and hepatic-β oxidation, resulting in accumulation of liver fatty acids and cholesterol, intensive hepatic inflammation and fibrosis. MCDD-induced model better mimicks the pathological features and mechanisms of severe human NASH. Thus, it is appropriate for assessing therapeutic drugs in NASH. Treatment with recombinant feline hepatocyte growth factor (HGF) has been shown to suppress the progression of NASH in a choline-deficient amino acid-supplement diet-induced mouse model [24]. However, MCDD leads to neither weight gain nor insulin resistance that fails to recapitulate the pathophysiology in human [25,26].

Fructose consumption has been linked to NASH and increased hepatic fibrosis [27]. Findings obtained with C57BL/6 mice fed with a high-fat, high-fructose diet (55% fructose and 45% sucrose) suggested that fructose consumption is required for the progression of liver fat deposition to fibrogenesis [22]. A NASH mouse model with rapid

Table 1
Mouse models used in NAFLD/NASH/HCC research.

Model	Features				
	Obesity	Steatosis	NASH	Fibrosis	HCC
Dietary models					
HFD	Y	Y	Y	Y	N
HFD + DEN	Y	Y	Y	Y	Y
HFD + STZ	Y	Y	Y	Y	Y
MCDD	N	Y	Y	Y	N
HF + HFD	Y	Y	Y	Y	N
Genetic models					
Specific *pnpla3*p.Ile148M	N	Y	N	N	N
Specific *tm6sf2*	N	Y	N	N	N
pten knockout	N	Y	Y	Y	Y
pemt knockout + MCDD	N	Y	?	?	?
agpat2$^{-/-}$	N	Y	?	?	?
bscl2$^{-/-}$	N	Y	?	?	?
Hyperphagic models					
db/db	Y	Y	N	N	N
db/db + MCDD	Y	Y	Y	Y	?
db/db + DEN	Y	Y	Y	?	Y
ob/ob	Y	Y	N	N (resistance)	N
ob/ob + HFD/MCDD	Y	Y	Y	N (resistance)	N
Mc4r$^{-/-}$ + HFD	Y	Y	Y	Y	Y
foz/foz mice + HFD	Y	Y	Y	Y	N
Circadian alteration triggered models					
cry1$^{-/-}$ + cry2$^{-/-}$	?	Y	?	?	Y (11.7%)
per1−/− + per2−/−	?	Y	Y	Y	Y (12.5%)
alb^{cre} + bmal1$^{fl/fl}$	?	Y	Y	Y	Y (11.53%)
car$^{-/-}$	?	Y	N	N	N
fxr$^{-/-}$	?	Y	Y	Y	Y (29.2%)

HFD: high-fat diet; DEN: diethyl-nitrosamine; STZ: streptozotocin; MCDD: methionine-choline deficient diet; HF: high-fructose; NASH: non-alcoholic steatohepatitis; HCC: hepatocellular carcinoma; fl/fl: flanked loxp; car: constitutive androstane receptor; fxr: farnesoid x receptor; Y, yes; N, no;?, insufficient data.

progression of steatohepatitis, extensive fibrosis and HCC has been established by feeding a western diet (containing 21.1% fat, 41% sucrose and 1.25% cholesterol in the food and 23.1 g/L D-fructose and 18.9 g/L D-glucose in the drinking water) combined with CCl4, which closely replicates the transcriptomic hallmarkers of human NASH [28].

The mixed diet model has been proposed for overcoming the limition of mono-diet-induced mouse model. For example, C57BL/6 mice fed a choline-deficient and L-amino acid-deficient diet develop liver injury and HCC [29]. HFD combined with streptozotocin induces hepatic steatosis, inflammation, fibrosis and eventually HCC in C57BL/6 mice [30]. The degree of hepatic steatosis and fibrosis depends on multiple factors, in particular the mouse strain [22]. The disease progression also varies greatly across different strains. For example, HMDP mice develop hepatic steatosis without fibrosis but BXD19/Ty mice develop liver fibrosis fed with the same western diet [31].

2.3. Genetic mouse models

Genetic mouse models mimicking different disease stages can help to mechanistically understand the development and progression of NAFLD/NASH-related HCC. Genetic models are mainly based on mutations associated with NAFLD predisposition. Patatin-like phospholipase domain-containing 3 (*PNPLA3*) gene encodes a membrane-bound enzyme expressed on the surface of lipid droplets and the smooth endoplasmic reticulum. PNPLA3 acts as a lysophosphatidic acid acyltransferase, and the polymorphism of p.Ile148Met increases its activity to induce the development of hepatic steatosis [32]. The p.Ile148Met variant in human *PNPLA3*, found in around 20% of the population, is associated with NAFLD progression [33]. Over-expressing *pnpla3*p.Ile148Met exhibits higher level of triglyceride and increased synthesis of fatty acid in mouse [34].

A variant of the transmembrane 6 superfamily member 2 *(TM6SF2* rs58542926) gene has been identified to confer susceptibility to NAFLD [35]. TM6SF2 is a membrane-bound protein located on the endoplasmic reticulum. Selective knockdown of *tm6sf2* in mouse liver causes increased hepatic triglyceride content and reduced VLDL secretion [35]. Though the precise function of TM6SF2 protein in NAFLD is still unknown, it is predicted to act as a lipid transporter and may interact with proteins involved in intestinal absorption [36].

Another NAFLD related gene is hepatocyte-specific phosphatase and tensin homolog (*PTEN*), known as a tumor suppressor for its lipid phosphatase activity and is important for preventing oncogenesis in the liver. *Pten*-deficient mice develop features similar to human NASH and NASH-related HCC [37]. A study has reported that liver tumors are present in 83% male and 50% female *pten*-deficient mice [38].

Polymorphisms of PEMT are associated with NAFLD in humans, and the *PEMT* gene is involved in phosphatidyl choline synthesis. *Pemt*-deficient mice fed with MCDD feature severe hepatic steatosis [39], which can be partially recovered by choline supplementation [40]. In addition, another two murine genetic models of lipodystrophy, *agpat2*$^{-/-}$ and *bscl2*$^{-/-}$ mice, have also been described of displaying hepatic steatosis [41,42].

2.4. Hyperphagic mouse models

Hyperphagic mouse models, such as db/db and ob/ob mice, have been described to develop NAFLD and NASH [43,44]. The db/db mice could also develop HCC when treated with diethylnitrosamine (DEN) at age of 13–15 days [45]. The db/db mouse model is leptin resistant, harboring a splice site mutation that abolish the expression of leptin receptor [43,46]. The db/db mice alone are good models of NAFLD but not NASH, and NASH can be induced by adding a second hit with an MCDD or trans-fat intake. The ob/ob mice are leptin deficient. They possess functional leptin receptor, but produce truncated and non-functional leptin. Given an *ad libitum* diet, ob/ob mice develop obesity, insulin resistance, hyperglycemia and hepatic steatosis [47]. The ob/ob

mouse rapidly develops NAFLD and presents early features of NASH at 20 weeks [46,48]. Second insults, such as MCDD and HFD, are needed to trigger steatohepatitis in ob/ob mice [49]. It has been reported that db/db mice show more severe NAFLD than ob/ob mice [43,44]. The main limitation of db/db and ob/ob mouse is that the etiology of obesity, for congenital leptin deficiency and leptin resistance resulted from gene mutations, is rare in obese human.

Melanocortin 4 receptor(Mc4r) ablated mice feature hyperphagic obesity without pathologically suppressed leptin levels. This model develops steatosis, exuberant NASH with established fibrosis after 20 weeks, and HCC by one year when exposed to HFD [50]. Mice with a mutated *alms1* gene (foz/foz mice) are morbidly obese and hyperphagic. The *alms1* gene plays a role in intracellular transport and appetite regulation. When studying NAFLD and progression to NASH, it is combined with HFD [51].

2.5. Circadian alteration triggered models

Circadian rhythm is a timekeeping system that regulates various metabolic pathways [52]. Chronic circadian disruption, also termed as "social jet lag", among night-shift workers and individuals suffering from sleep dyspnea has been identified as a common risk factor of obesity, metabolic disorders, NAFLD, and cancer including HCC [53–56].

Circadian homeostasis in mammalian is maintained by a central molecular clock, which is operated by circadian genes [57]. *Clock* encodes a bHLH (basic helix-loop-helix)-PAS (Period-Arnt-Sim) protein that regulates circadian rhythms [58]. Another two circadian genes, *Period* (including three homologs, *Per1, Per2* and *Per3*) [59] and *Bmal1* [60], and their downstream targets *Cryptochrome* (*Cry1* and *Cry2*) constitute a negative feedback loop, which generates the circadian rhythmicity [61].

As liver is a major metabolic hub, hepatic functions must adapt to the rhythmically changes of environment. The circadian clock is involved in regulation of hepatic triglyceride accumulation, inflammation, oxidative stress [62] and mitochondrial dysfunction [63], thus related to the pathogenesis of NAFLD/NASH [6]. Chronic circadian misalignment is sufficient to disrupt the liver clock and induce spontaneous NAFLD-related HCC in wide-type mice independent of dietary, exogenous genotoxic stress, or germline gene mutations [64]. Consistently, genetic ablation of these clock genes can also result in NAFLD and HCC [64]. The circadian alteration triggered models could closely mimic the spontaneous progression from NAFLD to NASH, and then fibrosis and HCC.

3. Advancing the mechanistic understanding of NAFLD, NASH and HCC

The "two-hit" model suggests that after a first hit (lipid accumulation), a second hit (*e.g.* oxidative stress, cytokines or DNA damage) is needed to develop NASH [65]. While the exact mechanisms that promote HCC development in NASH/NAFLD patients are complex. The pathogenesis of this disease has recently been recognized as multifactorial, involving lipid accumulation, mitochondrial dysfunction, inflammatory cytokines, immunologic mediators and gut-derived microbial components [66]. The currently availabe models have advanced our mechanistic understanding in this respect (Fig. 2).

3.1. Lipid accumulation and mitochondrial dysfunction in the liver

Lipid accumulation in liver is a crucial mediator in the pathogenesis of NAFLD. Increased lipid accumulation arises from abnormal lipolysis within peripheral adipose tissue, dietary and *de novo* hepatic lipogenesis [67], which causes excessive production of saturated and monounsaturated free fatty acids (FFAs) [68]. Serum FFAs are the contributors to liver lipid content. In insulin-resistant individual, which is commonly found in NAFLD, insulin is unable to suppress lipolysis, and then leads to release of FFAs into the portal circulation [69]. Thus, the esterification rate of liver is directly proportional to the amount of FFAs being delivered [70]. NAFLD-like phenotype could be developed by treating cell lines, ESC-derived HLC or liver organoids with monounsaturated and/or saturated FFAs *in vitro* [9,10,14,18].

NASH is a disease closely related to mitochondrial dysfunction. Fatty acids are effective activators of peroxisome proliferator activated receptors (PPARs), which is the regulator of liver mitochondrial metabolism [71]. Activated PPARs cause mitochondrial dysfunction and increased oxidative stress, leading to production of mitochondrial-derived ROS [6]. Under oxidative stress, the imbalance between ROS production and antioxidants results in lipid peroxidation, which generates toxic aldehyde byproducts. Together with ROS, these products cause damage to intracellular organelles, induce cell death and activate fibrogenic hepatic stellate cells [72]. Meanwhile, mitochondria-derived ROS can act as intrinsic danger signals and triggers of inflammatory responses, including macrophage activation and secretion of cytokines (*e.g.* TGF-β, TNF-α and IL-6), and finally leads to the activation of TRAIL signaling and hepatocyte apoptosis [73].

Many metabolic processes are regulated by circadian clocks [74]. PPARα is a direct transcriptional target of *BMAL1* and *CLOCK* [75,76]. $Bmal1^{KO}$ and $clock^{mutant}$ mice display abolished oscillation and decreased expression of *pparα* in liver; whereas $ppar\alpha^{KO}$ mice show altered oscillation of *per3* and *bmal1* [77]. The $ppar\alpha^{hep-/-}$ mice induced by MCDD and HFD develop severe NAFLD [78], confirming that hepatocytic PPARα deletion is sufficient to promote steatosis. Thus, PPARα has been recoganzied as a drug target for NAFLD. Currently, a phase III trial of PPARα/δ agnoist (elafibranor) is under evaluation in 2000 NASH patients [79].

3.2. Inflammatory cytokines in NAFLD-related HCC

Lipid accumulation increases the production of inflammatory cytokines through mechanisms involving mitochondrial-derived ROS [71,73]. Inflammatory cytokines including IL-6, TNF-α, leptin and adiponectin are out of balance and play pivotal roles in the progression of NAFLD/NASH to HCC [80,81]. Elevated IL-6 level has been observed in NASH patients [82]. As an important contributing factor to tumorigenesis, IL-6 can be induced by both TNF-α and IL-1. The $IL6^{-/-}$ male mice develop much fewer HCC tumors than wide type mice when fed on normal chow [83]. IL-6 stimulates cell proliferation and tumor progression in HCC by activating the JAK/STAT pathway [82,84]. In addition to IL-6, the dramatic increase of TNF-α has also been observed in obese and NASH mice [82]. TNF-α is a pro-inflammatory cytokine that regulates cancer cell differentiation, metastasis and tumor angiogenesis. HFD increases the expression of TNF-α, which elevates the levels of TLR4 and IL-8, and is positively associated with the presence of NASH [85]. The absence of either IL-6 or TNF-α in tumor-bearing mice reduces HFD induced hepatoseatosis and steatohepatitis [82].

Adiponectin is an anti-inflammatory adipokine inhibiting angiogenesis *via* modulating apoptosis. Adiponectin can suppress the expression of TNF-α and induce the expression of anti-inflammatory genes (*e.g.* IL-10 and IL-1 receptor antagonist) by eliciting AMPK signaling [86]. $Adiponectin^{-/-}$ mice showed high level of TNF-α mRNA expression in adiponectin tissue and high TNF-α protein concentration in the circulation [81]. Serum adiponectin level is significantly diminished in NASH patients [87], while patients with decreased adiponectin have a high risk of HCC development [88]. Mechanistically, adiponectin induces HCC cell apoptosis *via* activation of caspase-3 and phosphorylation of JNK. A meta-analysis including 698 controls and 1545 patients with NAFLD has demonstrated that hypoadiponectinemia is a prominent feature of NASH patients [89]. Leptin regulates appetite by targeting LEPRb in neurons. The action of leptin is involved in the increased expression of TNF-α and IL-6 [90], and the activation of JAK [91]. Patients with simple steatosis and NASH have higher serum level

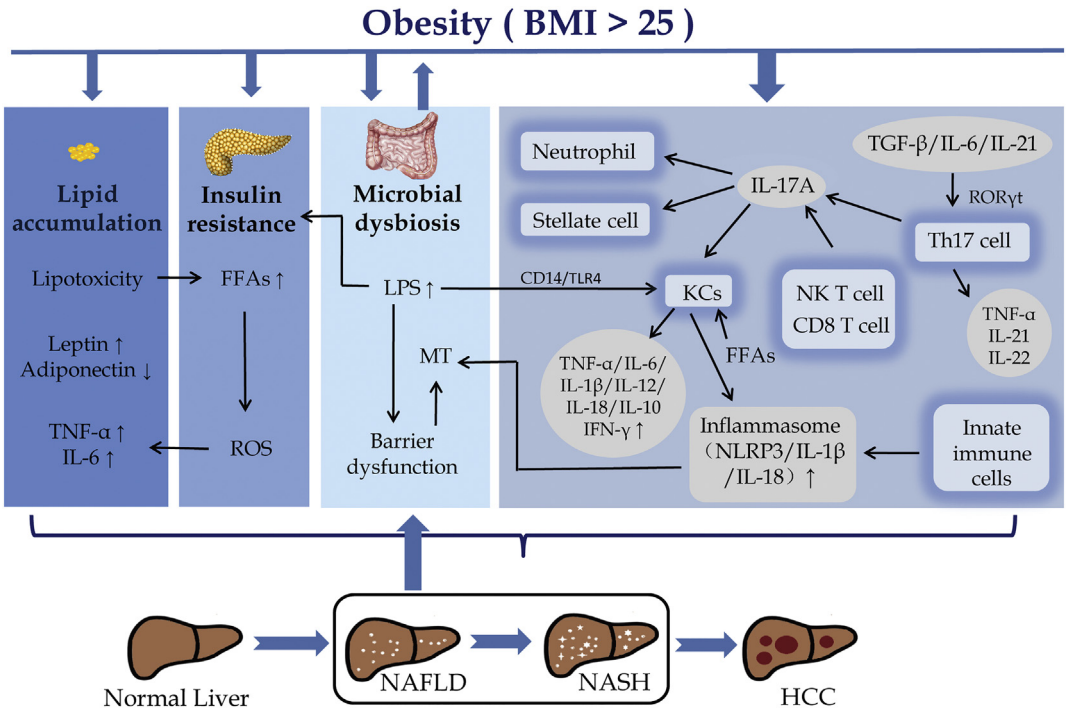

Fig. 2. Risk factors and proposed mechanisms for NAFLD/NASH to HCC development. The development of NAFLD and NASH-related HCC is multifactorial. According to estimates, 70–80% obese patients suffer from NAFLD. BMI: body mass index; FFAs: free fatty acids; IFN-γ: interferon-γ; IL: interleukin; KCs: kupffer cells; LPS: lipopolysaccharide; MT: microbial translocation; NLRP3: Nod-like receptor proteins; ROS: reactive oxygen species; RORγt: orphan nuclear receptor; TGF-β: transforming growth factor-β TLR4: Toll-like receptor-4; TNF-α: tumor necrosis factor-α; Th17 cell: T helper 17 cell.

of leptin. This is associated with increased severity of NAFLD and HCC [92]. Adiponectin treatment suppresses leptin-induced proliferation and invasion of HCC cells [93].

3.3. Immunologic mediators in NAFLD-related HCC

As a centrally immunological organ, liver can rapidly respond to circulating antigens, tissue damages and pathogen invasions. Kupffer cells (KCs), accounting for 20–35% of non-parenchymal cells in the liver, are strongly activated in NAFLD. Accumulation of hepatic lipids may result in impaired sinusoidal perfusion, and subsequently engage KCs response [94]. Meanwhile, FFAs, cholesterol and lipopolysaccharide (LPS) can directly activate KCs [95]. Activation of KCs leads to the up-regulation of TNF-α, IL-1β, IL-6, IL-12, IL-18, IL-10 and IFN-γ, which promote liver inflammation and NAFLD progression [96]. Destruction of KCs *via* liposome-encapsulated clodronate reduces inflammation and attenuates histological appearance of hepatic steatosis in MCDD mice [97]. Thus, KCs have been proposed as an attractive therapeutic target for NASH.

Th17 cells (a subset of CD4 T cells) and their production of IL17A have been implicated in NAFLD/NASH pathogenesis [98,99]. The combination of TGF-β, IL-6 and IL-21 induce the expression of RORγt, which is necessary for the development of Th17 cells in mice [100,101]. Activated Th17 cells secrete IL17A, IL21, IL22 and TNF-α to promote inflammation in liver. Suppressing Th17 cell differentiation by blocking RORγt prevents NASH and HCC development [99]. Neutralization of IL17 in C57BL/6 mice fed with HFD improves liver function, and attenuates steatosis and inflammation [102].

As multi-protein complexes modulating a spectrum of signals to secrete pro-inflammatory cytokines IL-1β and IL-18, inflammasomes contribute to the pathogenesis of NAFLD. The expression of inflammasome complexes (including Nod-like receptor proteins (NLRPs), caspase-1, IL-1β and IL-18) is increased in human NAFLD and NASH [103]. Innate immune cells, in particular KCs, possess the functional inflammasome machinery in the liver [73]. KCs promote hepatic steatosis *via* IL-1β dependent suppression of pparα activity in HFD-induced mice [104]. Diet-induced NAFLD mice exhibit improved hepatic function and decreased IL-1β and IL-18 expression after ablation of NLRP3 (NLRP3$^{-/-}$ mice) [105]. The activation of NLRP3 inflammasome is required for fibrogenesis in NASH [106]. However, the development of hyperphagia, obesity and insulin resistance has been reported in both IL-18$^{-/-}$ and ASC$^{-/-}$ (ASC, a component of NLRP3 inflammasome) HFD mice [107]. These discrepancies might be due to the different roles of multiple inflammasome complexes in various metabolic processes.

The interactions bewteen different types of immune cells are likely very important. Mice deficent in T cells or NK cells fail to develop fructose induced NAFLD; whereas immunocompetent mice and mice with B cell deficiency develop NAFLD [108]. CD8 T cells and NK T cells drive NASH and HCC development through interactions with hepatocytes in a choline-deficent high-fat diet mouse model, which recapitulats key features of human NASH and HCC [109]. Dysregulation of lipid metabolism in NAFLD mice causes a selective loss of intrahepatic CD4 T but not CD8 T cells, leading to accelerated hepatic carcinogenesis [110]. In patients, the complexed interactions of the immune system in NAFLD/NASH have been explored, but remain largely elusive [111–113].

3.4. Gut microflora dysregulation in NAFLD-related HCC

Portal blood flow links the gut and liver. The liver exposes to a multitude of gut metabolites and food products. Usually, bacterial molecules cross the mucosa only in trace amounts and enter the portal blood, but are cleared in the liver. This process relies on a balance between the barrier functions of the gut and the detoxification ability of the liver [114,115]. Disruption of tight junctions leads to barrier dysfunction, resulting in microbial translocation, and activation of immune response [116]. This acts as a possible inducer of necro-inflammatory lesions and severe fibrosis in NAFLD [117]. The link between gut dysbiosis and NAFLD has been observed in both animal and human

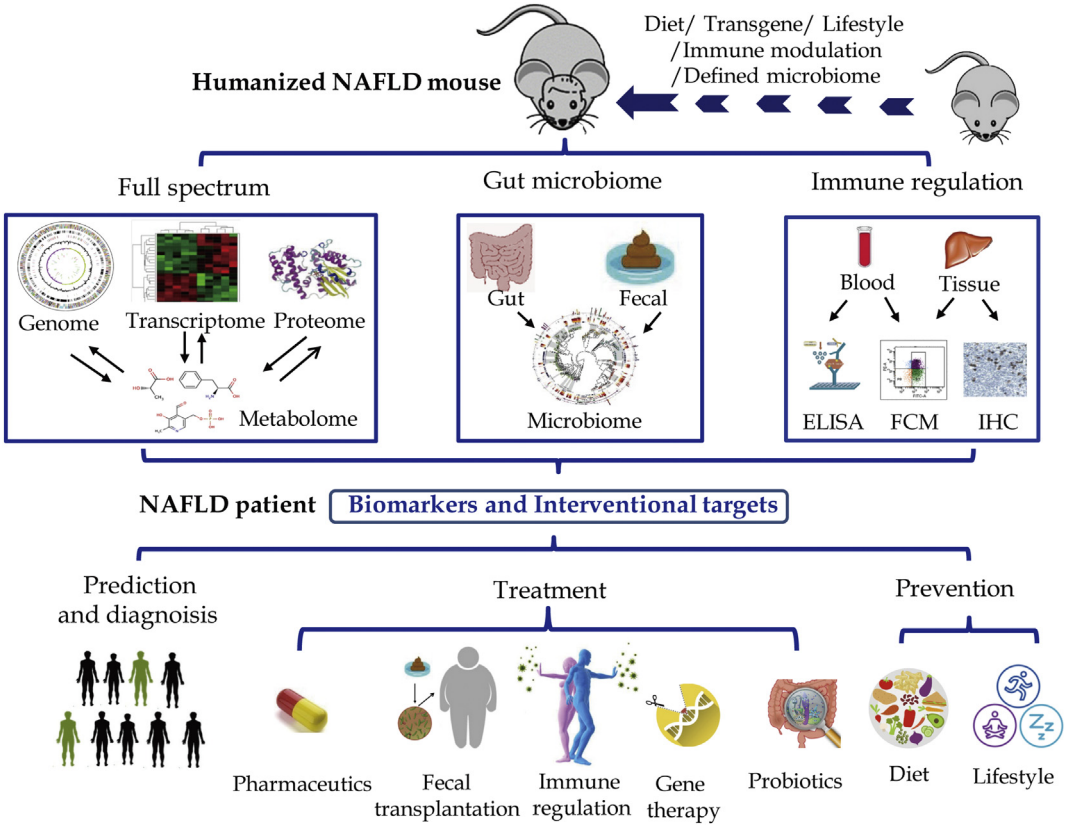

Fig. 3. Perspectives of prediction, diagnosis, prevention and treatment for NAFLD/NASH/HCC. The joint efforts from experimental to clinical research shall eventually expand our mechanistic understanding of this disease, and ultimately lead to the effective approaches for prediction, prevention and treatment. The advanced mouse models can be established by the combination of diet, genetic engineering, immune-deficent, defined microbiome, life style modulation and humanization. These models that recapitulate the disease features in human can be used for biomarker discovery, and development of new diagnosis, prevention and treatment strategies. ELISA: Enzyme linked immunosorbent assay; FCM: Flow cytometry; IHC: Immunohistochemistry.

[118,119]. Overgrowth of gut microbiota has been reported in NASH patients. In turn, NAFLD and NASH may cause gut barrier dysfunction, though the mechanisms remain elusive [120].

The products of gram-negative bacteria are also involved in NAFLD progression. For instance, gut derived LPS may activate the innate immune system through CD14 and TLR4 signaling, and trigger the secretion of pro-inflammatory cytokines and generation of oxidative stress [121]. As a marker of LPS activity, LPS binding protein has been found to be elevated in NAFLD patients and correlate with the stage of fibrosis [122]. The gut microbiota is associated with HCC progression through increasing LPS levels and shaping a pro-inflammatory microenvironment in liver. Treatment of antibiotics inhibits DEN/CCL4 induced liver tumor initation and grown in mice. Chronic treatment with a low, non-toxic dose of LPS results in a significant increase of the number and size of HCC tumors in mice [123].

The link among inflammasomes, gut microbiota and NAFLD has been demonstrated. Inflammasomes NLRP6 and NLRP3 (through activation of IL-18) negatively regulate NAFLD/NASH progression and cause metabolic syndrome *via* the modulation of gut microbiota [124]. Inflammasome deficiency has been linked to the alterations of gut microflora [125]. NLRP3 inflammasome is activated by microbial pathogen-associated molecules and therefore involves in NAFLD progression [126].

4. Conclusion and perspective

The application of proper experimental models is essential to demonstrate the causality of the disease. The aforementioned models have greatly facilitated the understanding of the development and progression from NAFLD to NASH and HCC, but have their specific limitions. No existing model displays the entire spectrum of NAFLD/NASH phenotypes as encountered in clinical practice.

The current *in vitro* models mainly capture the process of lipid accumulation in liver cells. However, the observation and measurement of intracellular lipid accumulation is much more straightforward in cell culture compared to animal models. This is convenient for assessing the therapeutic effects, in particular high-throughput screening of medications. The advance of liver organoids and 3D bio-printing technologies will likely take a step further that allow the modeling of multiprocesses of the disease, the interaction with other cell types, and the heterogenesities of individual patients. Specifically, the generation of liver organoids from NAFLD/NASH patients will likely better represent the disease characteristics. These models can be applied to find therapeutic targets through transcriptome or metabolome analysis, or to develop personalized treatment.

Mouse models have unique advantages in elucidating the pathogenesis and assessing therapeutic interventions. The ideal mouse model is expected to develop inflammation and fibrosis, but also respond to therapeutic interventions in the similar manner as in patients. There are several limitations for the current mouse models and their usefulness remains under debate [127]. For instance, population studies have identified polymorphisms in *PNPLA3* gene as important determinants of NAFLD progression in human. However, there is no discernible difference between *pnpla3*$^{-/-}$ and wild type mice [128]. This may be due to the distinct expression patterns that *pnala3* has the highest expression level in white adipose tissue of mice but in the liver of human [127].

To enhance their compatibility and clinical relavence, improved mice models are proposed to be generated with the combination of diet induction, genetic engineering, immune regulation, defined microbiome and life style modulation, whenever necessary. The combination

of genetic background, for instance a stable isogenic cross between C57BL/6 J (B6) and 129S1/SvImJ (S129) mice, results in better recapitulating the key features of NASH-associated HCC [129]. Humanization in mice is an attratcive approach. Transgenic mice overexpressing the human *PNPLA3-148 M* (a NAFLD suceptible variant) has been used to define its functions [130]. Chimeric mice with humanized liver, and even with reconstitution of the human immune system, have been widely used for studying hepatitis virus infections and drug toxicity [131]. These mice deserve to be explored for modeling NAFLD/NASH.

We expect that clinical and population-based association studies will continuously unveil new molecules and pathways, and importantly other extrinsic factors including environmental, life style and social changes that are associated with NAFLD/NASH/HCC in human. Using the right experimental models shall transform these clinical and population-based observations into new mechanistic insights. These knowledge in turn provide the basis for solving the contemporary challenges in the clinic, including identification of predictors and biomarkers, and development of new prevention and treatment strategies for combating NAFLD/NASH/HCC (Fig. 3).

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Conflicts of interest

The authors declare no conflict of interest. This manuscript has been read and approved by all authors, and is not submitted or under consideration for publication elsewhere.

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