REVIEW ARTICLE

Bile acid and receptors: biology and drug discovery for nonalcoholic fatty liver disease

Ting-ying Jiao[1], Yuan-di Ma[1,2], Xiao-zhen Guo[1], Yun-fei Ye[1,2] and Cen Xie[1,2]

Nonalcoholic fatty liver disease (NAFLD), a series of liver metabolic disorders manifested by lipid accumulation within hepatocytes, has become the primary cause of chronic liver diseases worldwide. About 20%–30% of NAFLD patients advance to nonalcoholic steatohepatitis (NASH), along with cell death, inflammation response and fibrogenesis. The pathogenesis of NASH is complex and its development is strongly related to multiple metabolic disorders (e.g. obesity, type 2 diabetes and cardiovascular diseases). The clinical outcomes include liver failure and hepatocellular cancer. There is no FDA-approved NASH drug so far, and thus effective therapeutics are urgently needed. Bile acids are synthesized in hepatocytes, transported into the intestine, metabolized by gut bacteria and recirculated back to the liver by the enterohepatic system. They exert pleiotropic roles in the absorption of fats and regulation of metabolism. Studies on the relevance of bile acid disturbance with NASH render it as an etiological factor in NASH pathogenesis. Recent findings on the functional identification of bile acid receptors have led to a further understanding of the pathophysiology of NASH such as metabolic dysregulation and inflammation, and bile acid receptors are recognized as attractive targets for NASH treatment. In this review, we summarize the current knowledge on the role of bile acids and the receptors in the development of NAFLD and NASH, especially the functions of farnesoid X receptor (FXR) in different tissues including liver and intestine. The progress in the development of bile acid and its receptors-based drugs for the treatment of NASH including bile acid analogs and non-bile acid modulators on bile acid metabolism is also discussed.

Keywords: bile acids; nonalcoholic fatty liver disease; nonalcoholic steatohepatitis; drug target; Farnesoid X receptor; G protein-coupled bile acid receptor

INTRODUCTION

Nonalcoholic fatty liver disease (NAFLD) has become the principal aetiology of chronic liver diseases around the world [1], with an overall global prevalence of 25% [2]. Non-alcoholic steatohepatitis (NASH), a subtype of NAFLD, is expected to affect ~1.5% to 6.45% of the general population [2]. NASH is characterized as steatosis with inflammation infiltration and hepatocyte ballooning, with or without fibrosis, leading to associated complications such as cirrhosis, hepatocellular cancer (HCC) and liver-related mortality [3–5]. The prevalence of NASH is estimated to rise by 63% between 2015 and 2030 [6], which puts a great burden on global healthcare. In general, lifestyle modifications without drug intervention, such as calorie-restricted diets or physical exercise, can improve hepatic steatosis and liver histology in NAFLD patients caused by western-style diets or sedentary lifestyles [7]. However, the influence of changing lifestyle behaviors in reversing liver fibrosis and injury in NASH is limited [8]. Therefore, efficacious drugs are needed to arrest or reverse the disease progression.

The "two-hit hypothesis" and "multiple-hit hypothesis" were put forward, which are generated by abnormal lipid accumulation and the development of inflammation induced by sequential hepatotoxic injuries within hepatocytes or extrahepatic tissues [9], to explain the complex pathogenesis of NASH. In western countries, NAFLD is closely related to a series of metabolic dysfunction (e.g., insulin resistance (IR), increased body mass index, hypertension, dyslipidemia, obesity and type 2 diabetes) [10]. On this basis, the recent proposal of a more appropriate term metabolic dysfunction-associated fatty liver disease (MAFLD) helps define NAFLD with metabolic syndrome [11], so as to better understand its mechanism. Current therapeutic targets for NASH are mainly focused on agonists for nuclear receptors involved in steatosis, inflammation or fibrogenesis such as farnesoid X receptor (FXR), thyroid hormone receptor-β (TRβ) and peroxisome proliferator-activated receptor α (PPARα), as well as analogs of enterohepatic hormones including glucagon-like peptide 1 (GLP-1) and fibroblast growth factor 19 (FGF19) and 21 (FGF21). However, the drugs approved to treat NASH patients is limited.

Over the last two decades, bile acids have been recognized as pivotal signaling molecules in NASH pathogenesis that enable fine-tuned gut-liver communication from the liver, the site of bile acid production, to the intestine, the site of nutrition sensing, to virtually throughout the body, where they exert their pleiotropic physiological functions. Bile acids are a group of amphiphilic molecules produced by hepatocytes from cholesterol and

[1]State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Chinese Academy of Sciences, Shanghai 201203, China and [2]University of Chinese Academy of Sciences, Beijing 100049, China
Correspondence: Cen Xie (xiecen@simm.ac.cn)
These authors contributed equally: Ting-ying Jiao, Yuan-di Ma.

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secreted through the bile ducts into the intestine and exert a prominent role in fat emulsification, as well as lipids and fat-soluble vitamins digestion and absorption after food ingestion. In liver, cholesterol is transformed to two primary bile acids, cholic acid (CA) and chenodeoxycholic acid (CDCA), which are then conjugated to glycine or taurine through bile acid synthetic pathways. They are further secreted into bile across the apical membrane of the hepatocytes, promoting the solubilization of cholesterol, and released into the small intestine under post-prandial status. Then bile acids are metabolized by the intestinal bacteria into multiple secondary bile acids such as lithocholic acid (LCA) and deoxycholic acid (DCA), most of which are reabsorbed into the bloodstream in the terminal ileum, then circulated to the liver and re-secreted into the bile [12]. Apart from the classical function as a fat emulsifier, bile acids also function as pivotal signaling molecules involving a series of biological functions such as metabolic homeostasis and immune response through activation of two main bile acid receptors, nuclear FXR and membrane G protein-coupled bile acid receptor 1 (GPBAR1 or TGR5), in humans and rodents [13, 14]. Disturbed bile acid homeostasis is considered to be related to many metabolic diseases, including NASH, rendering bile acids and their receptors potential targets for therapeutic intervention [15, 16].

Recent studies have demonstrated that some natural bile acids and their derivatives promote the development of NASH, while others have therapeutic effects on NASH [15, 17]. Several small molecules targeting bile acid metabolism, transport or bile acid receptors have been undergoing clinical trials for NASH treatment [17, 18]. In this review, the biology of bile acids and the advances in the functions of bile acid receptors will be briefly discussed, and recent progress on bile acid- or receptors-based drug discovery for NASH therapeutics will be highlighted.

Bile acid biology and physiology

Primary bile acid synthesis. Two main primary bile acids, CA and CDCA, are synthesized by hepatocytes via two pathways, including the classical pathway and the alternative pathway, respectively (Fig. 1). In the classical pathway, cholesterol is first hydroxylated by cholesterol 7α-hydroxylase (CYP7A1) to produce 7α-hydroxycholesterol. 7α-Hydroxy-4-cholestene-3-one (C4) is the downstream intermediate [19], C-12 hydroxylation of which by sterol 12α-hydroxylase (CYP8B1) produces CA. Otherwise, C4 is transformed to CDCA. In the alternative pathway, cholesterol side chain is hydroxylated by sterol 27-hydroxylase (CYP27A1) [20]. The oxysterol 7α-hydroxylase (CYP7B1) is in charge of the C-7α hydroxylation to synthesize CDCA in the alternative pathway [21]. It is noteworthy that rodent liver specifically expresses CYP2C70, that converts most CDCA to α-muricholic acid (α-MCA), which is then isomerized to β-MCA [22]. Therefore, α-MCA and β-MCA are also recognized as primary bile acids in rodents.

In liver, most bile acids are converted to conjugated forms (glycine- or taurine-) in the peroxisomes by bile acid-CoA synthetase (BACS) and amino acid N-acyltransferase (BAAT), which reduces their toxicity and increases the solubility. They are then secreted into the bile through the bile salt export protein (BSEP), facilitating their physiological effect as a cholesterol carrier and surfactant in the bile and intestine [23]. In addition to the abundant amidated bile acids, the liver also produces, to a minor extent, sulfates and glucuronides of bile acids via sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), and then are excreted to the bile or urine via multidrug resistance-related protein 2 (MRP2).

Secondary bile acid synthesis: transformation in the gut. After secreted into the intestine lumen, primary bile acids are transformed into secondary bile acids by bacterial enzymes (Fig. 1). In the distal ileum and colon, *Bacteroides*, *Clostridium*, *Lactobacillus*, *Enterococcus* and *Bifidobacterium* express bacterial

bile salt hydrolases (BSH) [24], which produce free bile acids that are further 7α-dehydroxylated by bacterial 7α-dehydroxylase and converted to the secondary bile acids (e.g. CA to DCA, CDCA to LCA). In humans, ursodeoxycholic acid (UDCA) is produced by 7α-OH isomerization (to 7β-OH) of CDCA, which are further dehydroxylated to LCA by gut bacteria. In humans and pigs, 6α-hydroxylase mediates the conversion of CDCA to the more hydrophilic hyocholic acid (HCA) in the liver, which is then dehydroxylated to hyodeoxycholic acid (HDCA) in the intestine. In rodents, most α-MCA and β-MCA can be epimerized to ω-MCA, and LCA can be 7β-hydroxylated to UDCA, or 6α-hydroxylated to HDCA, or 6β-hydroxylated to murideoxycholic acid (MDCA) in the liver [25]. Furthermore, the α-hydroxyl groups in bile acids are epimerized to carbonyl groups by 3α, 7α, and 12α-hydroxysteroid dehydrogenases (HSDHs) from gut bacteria, to produce 3-oxo, 7-oxo, and 12-oxo-bile acids, which are transformed to β-epimers, iso-bile acids, and epi-bile acids by 3β, 7β, and 12β-HSDHs [24]. It should be noted that in mouse liver, rather than other species, DCA and LCA can be hydroxylated to reform CA and CDCA by a newly identified enzyme cytochrome P450 2A12 (CYPA2A12) [26].

Bile acid enterohepatic circulation. The enterohepatic circulation of bile acids (Fig. 1) is defined as the process of bile acids flowing into the intestine via the bile duct, reabsorbed into the intestine and cycled to the liver though the portal vein [15]. Dietary intake provokes the delivery of cholecystokinin from the pancreas, thereby stimulating gallbladder contractions, ultimately releasing bile acids into the gastrointestinal tract. Bile acids promote nutrient absorption in the ileum and the majority of them are efficiently reabsorbed into the intestinal epithelium by entero-cytes via the apical sodium-dependent bile salt transporter (ASBT) in the terminal ileum [27]. By facilitation of ileal bile acid transporter protein (IBAT), bile acids are transported across the enterocytes and excreted into the portal blood by organic solute transporter-α and -β (OSTα and OSTβ) [28, 29]. The reabsorbed primary and secondary bile acids circulate via the portal vein to the hepatic sinusoids and are taken in by hepatocytes through Na$^+$-taurocholate co-transporting polypeptides (NTCP, conjugated bile acids) and organic anion transporters (OATPs, unconjugated bile acids), respectively [12, 30].

Bile acid pool and composition. The bile acid pool comprises the total bile acid content in the enterohepatic circulation, with 1% in liver, 10%–15% in gallbladder, and 85%–90% in gut, without bile acids in the systemic circulation [25]. Generally, the conjugated bile acids represent the large majority of bile acids found in the blood, liver, bile, and small intestine, while the largest proportion of free bile acids are found in the colon and feces. Taurine conjugates are the predominant ones in the bile acid pool of mice, rats, and dogs, whereas in humans, hamsters, and rabbits, the bile acid pool is composed of both glycine and taurine conjugates [31], which may be attributed to substrate specificity of BAAT and glycine and taurine availability. Moreover, the bile acid pool is composed of CA (about 40%), CDCA (about 40%), DCA (about 20%) in humans. In terms of conjugated bile acids, glycine-conjugated bile acids are approximately threefold of taurine-conjugated bile acids, while in mice, the bile acid pool comprises CA (about 60%) and α-plus β-MCA (about 40%) with more than 90% bile acids taurine-conjugated [25]. Intriguingly, hamsters have similar bile acid profiles as both humans and mice with high levels of MCAs as well as glycine conjugates, suggesting that hamster is a more ideal animal model to study bile acid-related pathogenesis and pharmacological interventions [32].

Bile acids possess both hydrophilic and hydrophobic surfaces, which makes these molecules detergent-like amphiphilic. In general, the number, location, stereo-configuration, and amidation of the hydroxyl groups on the steroid ring determine the hydrophilic features of bile acids and the solubility of
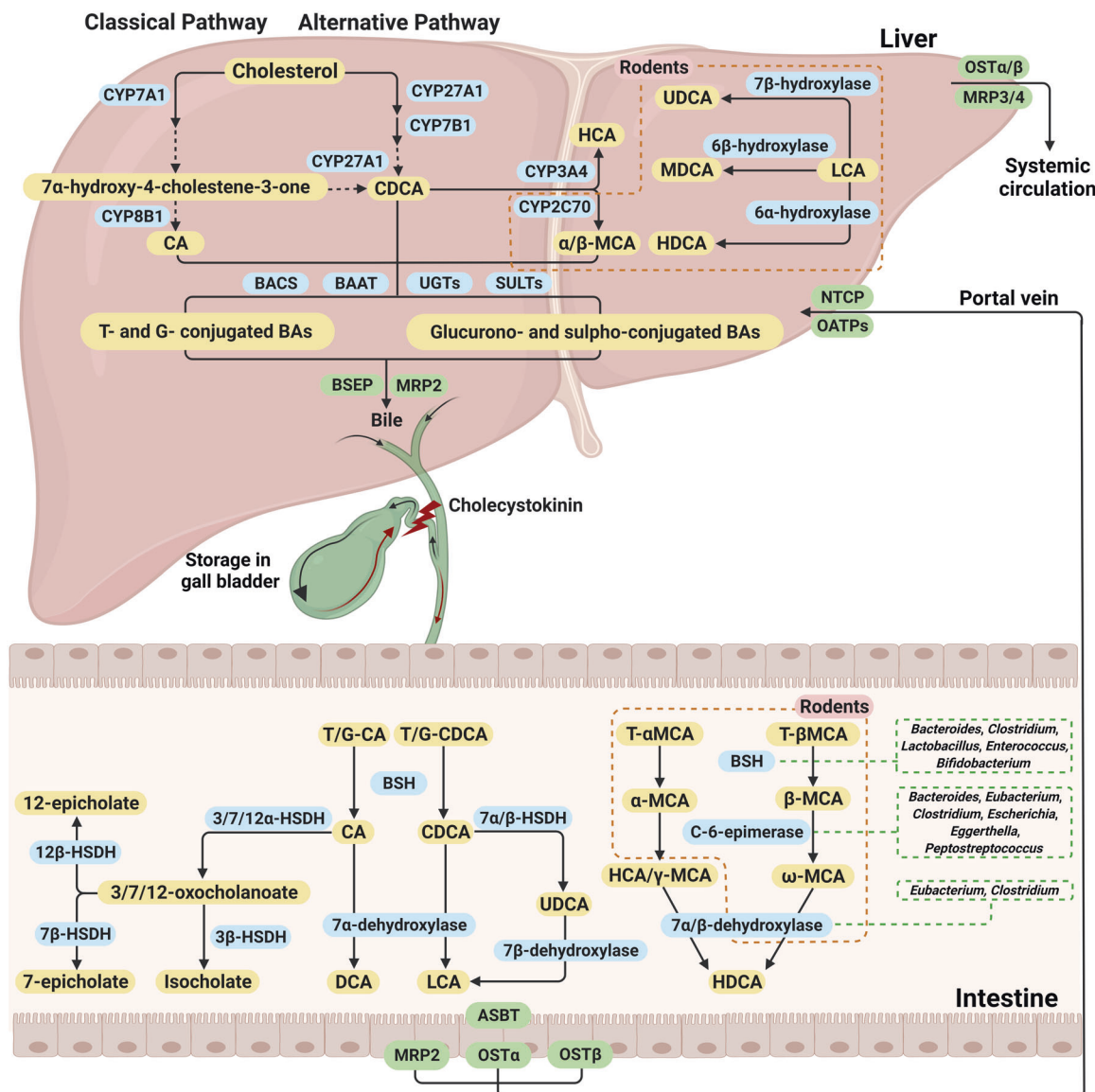
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Fig. 1 Bile acid synthesis and enterohepatic circulation. Hepatocytes produce primary bile acids via the classical and alternative pathways. The classical pathway starts with cholesterol 7α-hydroxylase (CYP7A1) and produces cholic acid (CA) and chenodeoxycholic acid (CDCA) through the action of sterol 12α-hydroxylase (CYP8B1) and sterol 27-hydroxylase (CYP27A1). The alternative pathway is initiated by CYP27A1 and produces CDCA through the action of oxysterol 7α-hydroxylase (CYP7B1). In rodents, CDCA can be mostly converted to α-muricholic acid (α-MCA) and β-MCA by a sterol 6β-hydroxylase (CYP2C70). Lithocholic acid (LCA) can be 7β-hydroxylated to ursodeoxycholic acid (UDCA), or 6α-hydroxylated to hyodeoxycholic acid (HDCA), or 6β-hydroxylated to murideoxycholic acid (MDCA). In pigs and humans, CDCA can be converted to hyocholic acid (HCA) by cytochrome P450 3A4 (CYP3A4) and then dehydroxylated to HDCA in the intestine. Most bile acids are conjugated to glycine (G) or taurine (T) via the action of bile acid-CoA synthetase (BACS) and amino acid N-acyltransferase (BAAT) and are secreted into the bile via bile salt export protein (BSEP). Meanwhile, hepatocytes produce sulphated (sulpho-) or glucuronidated (glucurono-) bile acids via sulfotransferases (SULTs) and UDP-glucuronosyltransferases (UGTs), which are secreted into the bile via multidrug resistance-related protein 2 (MRP2). The bile formed is stored in the gallbladder. After a meal, the release of cholecystokinin from the pancreas causes bile to be released into the duodenum. The conjugated primary bile acids are transformed to secondary bile acids by the action of intestinal bacterial bile salt hydrolases (BSH) and different dehydroxylases. β-MCA is differentially isomerized by C-6 to form ω-MCA, and then ω-MCA is 7α-dehydroxylated to form HDCA. CDCA is converted to UDCA by hydroxysteroid dehydrogenases (HSDHs), and then UDCA is converted to LCA by 7β-dehydroxylase. Gut bacterial 3α, 7α, and 12α-HSDHs epimerize the α-hydroxyl groups of bile acids to carbonyl groups to form 3-oxo, 7-oxo, and 12-oxo-bile acids, and then the carbonyl groups in oxo-bile acids are converted to isocholate, 7-epicholate and 12-epicholate by 3β, 7β, and 12β-HSDHs. At the end of the ileum, ~95% of the bile acids involved in the hepatic-intestinal circulation are reabsorbed by enterocytes into the intestinal epithelium via the apical sodium-dependent bile salt transporter (ASBT), transported across the enterocytes to the sinusoidal membrane, excreted into the portal vein via organic solute transporter-α and -β (OSTα and OSTβ) and MRP2 and transported back to the liver via the portal vein by the uptake action of Na$^+$-taurocholate co-transporting polypeptides (NTCP, conjugated bile acids) and organic anion transporters (OATPs, unconjugated bile acids). Bile acids enter the systemic circulation from hepatocytes via MRP3, MRP4, OSTα, and OSTβ. The figure was created with BioRender.com.

bile acid pool (Table 1), which is directly related to the fat-soluble nutrient absorption and bactericidal activity. The 6α-OH and 7α-OH bile acids are more hydrophobic and have higher bactericidal activities than their respective β-epimers and oxo-bile acids. Thus, the epimerization of CDCA to its β-epimer UDCA increases the solubility and decreases the toxicity of the bile acid pool. Moreover, taurine-conjugated bile acids are generally more hydrophilic and less toxic than the corresponding

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Table 1. Endogenous bile acids and their chemical structures

Amidation (BACS, BAAT)

Taurine: $NH_3^+(CH_2)_2SO_2O^-$
Glycine: $NH_3CH_2COO^-$

Sulfonation (SULT2A1, 2B8)

Glucuronidation (UGT1A3, 2B4, 2B7)

Sulfate: SO_3^-

Glucuronide

Hydrophobicity:
LCA > DCA > CDCA > CA > HDCA > UDCA > β-MCA > ω-MCA

Activity of FXR agonist:
CDCA > CA > LCA > DCA

Activity of TGR5 agonist:
LCA > DCA > CDCA > CA

Bile acid name	Abbreviation	C_3-R_1	C_6-R_2	C_7-R_3	C_{12}-R_4
Monohydroxy bile acid					
lithocholic acid	LCA	...OH	H	H	H
Dihydroxy bile acids					
chenodeoxycholic acid	CDCA	...OH	H	...OH	H
ursodeoxycholic acid	UDCA	...OH	H	OH	H
deoxycholic acid	DCA	...OH	H	H	...OH
hyodeoxycholic acid	HDCA	...OH	...OH	H	H
Trihydroxy bile acids					
cholic acid	CA	...OH	H	...OH	...OH
α-muricholic acid	α-MCA	...OH	OH	...OH	H
β-muricholic acid	β-MCA	...OH	OH	OH	H
ω-muricholic acid	ω-MCA	...OH	...OH	OH	H
hyocholic acid	HCA/γ-MCA	...OH	...OH	...OH	H
Oxo bile acid metabolites					
3-oxocholic acid	3-oxoCA	O	H	...OH	...OH
3-oxolithocholic acid	3-oxoLCA	O	H	H	H
7-oxocholic acid	7-oxoCA	...OH	H	O	...OH
7-oxochenodeoxycholic acid	7-oxoCDCA	...OH	H	O	H
12-oxocholic acid	12-oxoCA	...OH	H	...OH	O
12-oxodeoxycholic acid	12-oxoDCA	...OH	H	H	O

glycine-conjugated and free bile acids. As a result, the hydrophobicity of bile acids is ordered by LCA > DCA > CDCA > CA > HDCA > UDCA > β-MCA > ω-MCA as free bile acids, then their glycine conjugates, and last their taurine conjugates. Abnormalities in the size and/or composition of the bile acid pool might implicate the damage of bile acid metabolism induced by liver injury, bile duct obstruction, or gut dysbiosis.

Bile acids are also characterized as signaling molecules that regulating metabolic and inflammatory functions because they are able to interact with nuclear receptors such as FXR, PPARα, vitamin D receptor (VDR), as well as G protein-coupled receptor TGR5. The overall effect of bile acids on above mentioned receptors is determined by the balance between agonists and antagonists in the bile acid pool in each tissue they are expressed. Emerging evidences have suggested that the involvement of these bile acid-activated receptors during the development of NAFLD, which will be discussed below.

FXR controls bile acid homeostasis. FXR, the first identified bile acid-activated nuclear receptor, is considered as a predominant regulator of bile acid homeostasis [13]. Among all the major bile acid species, CDCA is the most potent FXR agonist, followed by CA > LCA > DCA [33], while glycoursodeoxycholic acid (GUDCA), taurochenodeoxycholic acid (TCDCA), tauroursodeoxycholic acid (TUDCA), and tauro-β-MCA (T-βMCA) were identified as FXR antagonists [34–37]. FXR is abundantly expressed in the gastrointestinal tract, including liver and intestine, and functions as a principal sensor of bile acids to mediate inhibitory feedback of bile acid synthesis and maintain low levels of intrahepatic bile acids to prevent cholestatic liver injury.

Two FXR-dependent pathways were proposed for the feedback regulation of bile acid synthesis (Fig. 2). In liver, excessive bile acids contribute to FXR activation and the induction of small heterodimer partner (SHP) consequently. Without DNA binding domain, SHP is considered to form heterodimers with several nuclear factors and inhibit their activity, including hepatic nuclear factor 4α (HNF4α) and liver receptor homolog 1 (LRH-1) that inhibit transactivation of downstream genes such as *CYP7A1* [38]

and *CYP8B1* [39], thereby repressing bile acid synthesis. In intestine, fibroblast growth factor 15/19 (*FGF19* in humans, *Fgf15* in rodents) is induced by FXR and thereby activates the hepatic fibroblast growth factor receptor 4 (FGFR4)/β-klotho complex, triggering extracellular signal-regulated kinase 1/2 (ERK1/2) and c-Jun-N-terminal kinase (JNK) signal, which results in the transcriptional repression of *CYP7A1* and bile acid synthesis [40]. Additionally, FXR have an important effect in regulating bile acid enterohepatic circulation. In liver, FXR suppresses the expression of bile acid uptake transporters NTCP and OATPs on the sinusoidal membrane, and induces BSEP that transports bile acids into the bile, thus maintaining bile acids in hepatocytes at very low levels. In intestine, FXR inhibits the expression of ASBT that mediates the reabsorption of bile acids into enterocytes, but induces IBAT, OSTα and OSTβ, thereby controlling bile acid reabsorption and secretion into the portal blood to prevent abundant bile acids accumulated in enterocytes, thus maintaining intestinal barrier function. Both hepatic and intestinal FXR work in concert to decrease the synthesis and reabsorption of bile acids in order to avoid cholestasis. Studies suggest that disturbed bile acid metabolism can result in the development of NAFLD by altering FXR signaling [41, 42].

Bile acid metabolism and NAFLD
The manifestation and pathogenesis of NAFLD. NAFLD is a spectrum of aberrant liver diseases from steatosis to steatohepatitis. Nonalcoholic fatty liver (NAFL) is the most benign one due to increased lipid accumulation within hepatocytes, also termed as hepatic steatosis. About 20%–30% NAFL patients might progress to NASH that is characterized by obvious lobular inflammation and hepatocyte ballooning beyond steatosis, which might ultimately progress to fibrosis, even cirrhosis [43]. Emerging evidences have demonstrated that the development of NAFLD is not driven by a single factor, but by whole-body metabolic dysregulation with a multitude of interlinked process (e.g., IR and lipotoxicity). Thus, it can be defined as the liver manifestation of a metabolic syndrome that is bound up with other metabolic disorders (e.g. obesity, type-2 diabetes and cardiovascular diseases) [10, 44].
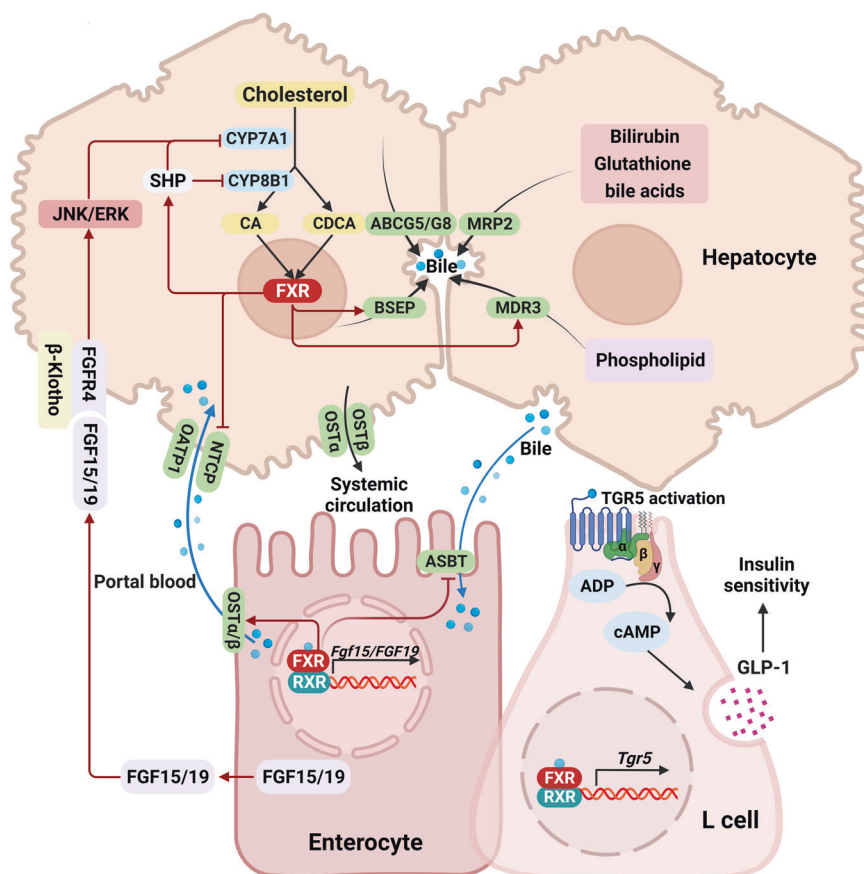
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Fig. 2 FXR and TGR5 in the enterohepatic circulation. Activation of hepatic farnesoid X receptor (FXR) by primary bile acids synthesized from cholesterol increases the expression of the small heterodimer partner (SHP), which inhibits CYP7A1 and CYP8B1 expression. At the same time, FXR inhibits NTCP to reduce bile acid uptake from circulation, induces BSEP to promote bile acid secretion into the bile, and induces multidrug resistance protein 3 (MDR3) to promote phospholipid secretion into the bile. Activation of intestinal FXR by bile acids increases the expression of fibroblast growth factor 15/19 (FGF19 in humans, FGF15 in rodents), induces the entry of these proteins into the liver via the enterohepatic circulation and acts on fibroblast growth factor receptor 4 (FGFR4)/β-klotho complex, thereby inhibiting the expression of CYP7A1. Meanwhile, FXR inhibits the expression of ASBT, but induces OSTα and OSTβ. In enteroendocrine L cells, FXR induces G protein-coupled bile acid receptor 1 (GPBAR1 or TGR5) to activate cyclic adenosine monophosphate (cAMP), which leads to secretion of glucagon-like peptide-1 (GLP-1) and stimulates insulin secretion from pancreatic cells. The figure was created with BioRender.com.

The primary "two-hit hypothesis" put forward decades ago was recently developed to a "multiple-hit hypothesis" to explain the pathogenesis of NASH (Fig. 3). Briefly, hepatic lipid input and output are disbalanced, leading to lipid accumulation within hepatocytes, which is considered as the "first hit". This makes liver more susceptible to other damages such as oxidative stress and hepatocyte apoptosis, triggering activation of hepatic stellate cells (HSCs) and increased inflammation generated by Kupffer cells, adipokines from adipocytes or metabolites from gut bacteria, followed by the deposition of extracellular matrix and fibrosis [45, 46]. These "multiple hits" act separately and drive the progression from NAFL to NASH. Notably, different from the "two-hit hypothesis", the current theory regards IR as the initial hit, which contributes to the increased free fatty acids (FFA) and sensitizes the liver to the hepatotoxic insults mentioned above [47]. A distinct-hit theory has recently emerged pointing out that pure fatty liver is separated from NASH. Furthermore, steatosis in NASH is a benign phenotype but not a factor that leads to inflammation and fibrosis [48]. These updated theories combine the timing and different paralleled pathogenic events, but not simply consider lipid accumulation as the driver of NASH, which is consistent with the absence of fatty liver in some NASH patients [48].

Role of bile acid metabolism in NAFLD. The disturbance of bile acid metabolism takes place during NAFLD progression and there

is a distinct serum bile acid pattern in NASH patients [42, 49], which is independent of obesity and diabetes [50]. To be specific, increased plasma concentrations of CDCA, DCA and UDCA were observed in NASH patients compared with those of healthy control [51]. In addition, plasma levels of glycocholate (GCA), glycodeoxycholate (GDCA), taurocholate (TCA), taurodeoxycholate (TDCA) and UDCA are higher in patients with NASH [42, 52, 53]. NAFL advancing to NASH features in an increase in the ratios of primary to secondary and conjugated to unconjugated bile acids [42, 54]. Certain CA conjugates showed a positive correlation with the histological signature of NASH, including TCA with steatosis and ballooning, GCA with lobular inflammation, as well as total conjugated CA and TCA with overall NAFLD activity [42]. The abundance of hepatic CA and the ratio of trihydroxylated bile acids to dihydroxylated bile acids were positively associated with the severity of inflammation [55].

Clinical studies have demonstrated that the occurrence of NAFLD is companied by disturbed bile acid homeostasis and its related signaling pathways. Among these findings, primary bile acids are generally observed increased, whereas secondary bile acids are decreased [42, 56]. The increased ratio of total CA to CDCA [42, 52] and the elevated serum C4 levels [57] are frequently observed in NASH patients. These data together implicate the inhibition of hepatic FXR-SHP axis, which leads to the induction of CYP7A1 [41, 42] and CYP8B1 [50]. There are conflicting results
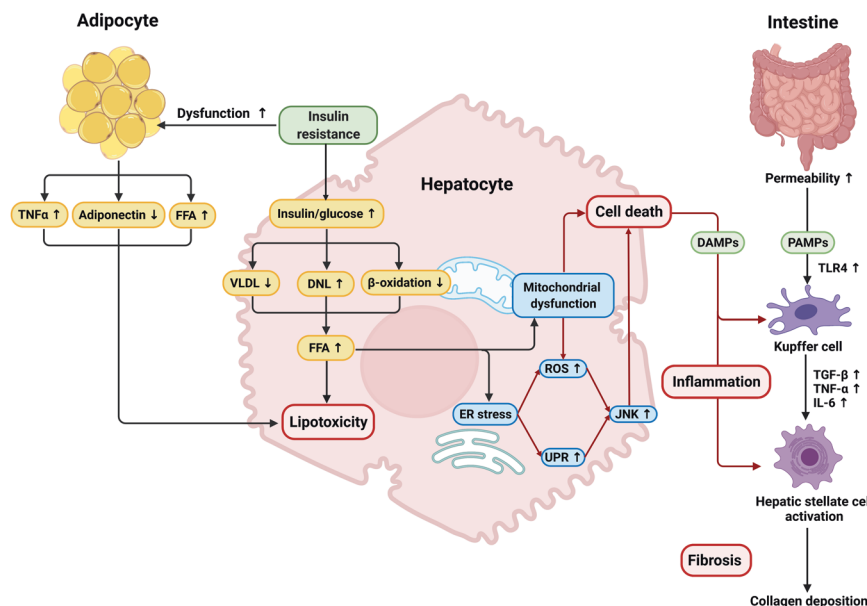
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Fig. 3   Metabolic factors for the development of NASH. Insulin resistance (IR) acts on adipose tissue and worsens adipocyte dysfunction, inducing lipolysis, release of adipokines and pro-inflammatory cytokines. In the liver, IR amplifies de novo lipogenesis (DNL) and reduces very low-density lipoprotein (VLDL) and β-oxidation. Increased hepatic free fatty acids (FFA) lead to mitochondrial dysfunction and endoplasmic reticulum (ER) stress, which leads to the production of reactive oxygen species (ROS) and activation of the unfolded protein response (UPR), and finally to the activation of c-Jun N-terminal kinase (JNK). Hepatic stellate cell can be activated directly by the accumulation of FFA in the liver, also known as damage-associated molecular pattern (DAMPs), or indirectly by IL-6, TNF-α and TGF-β secreted by Kupffer cells, leading to collagen deposition. Meanwhile, increased permeability of the small intestine leads to stimulation of Toll-like receptor 4 (TLR4) and activation of Kupffer cells by pathogen-associated molecular patterns (PAMPs) such as lipopolysaccharide (LPS), pro-inflammatory factors, etc., which subsequently activates an inflammatory cascade response. The figure was created with BioRender.com.

about the change of hepatic CA in NASH, with one study showing that intrahepatic CA is increased [55], but another two studies finding that levels of CA in liver tissues from NASH patients were significantly decreased [58, 59]. Considering that hydrophobic bile acids are strong FXR ligands, more hydrophilic metabolites in total hepatic bile acids of NASH patients may imply repressed FXR signaling [59]. In addition, FXR-mediated bile acid metabolism is influenced by feeding state, and the effect of food on subjects in different cohorts may also contribute to the variation in bile acid composition.

Patients with chronic liver disease of different etiologies (e.g., NASH, hepatitis B virus (HBV), hepatitis C virus (HCV), alcohol-induced liver disease (ALD) and primary biliary cirrhosis) also have different bile acid spectra, among which the highest ratio of 12α-hydroxylated (12α-OH) to non-12α-OH bile acids was observed in NASH patients [60]. As plasma bile acids have been proposed as NASH biomarkers, a recent study found that the elevation of bile acids was only seen in NASH patients with significant IR, limiting their use as biomarkers [61].

Role for bile acid receptors in metabolic disorders and NAFLD
FXR.
Lipid and glucose metabolism in liver: The effect of both hepatic and intestinal FXR on the physiological regulation of glucose and lipid metabolism has been extensively studied [62, 63]. A few human single nucleotide polymorphisms in the *FXR* gene are related to fasting hyperglycemia and FFA [64], while mutations in the *SHP* gene are correlated with obesity [65, 66] and type 2 diabetes susceptibility [67]. Specifically, liver FXR activation dampens *de novo* lipogenesis by repressing sterol regulatory element binding protein 1 (*Srebp1*) and the lipogenic genes fatty acid synthase (*Fasn*), acetyl-CoA carboxylase 1 (*Acc1*), stearoyl-Co A desaturase-1 (*Scd1*) [68], and also affects lipid transport by targeting lipoproteins such as phospholipid transfer protein [69] and apolipoprotein C-III [70]. Moreover, liver FXR promotes fatty

acid oxidation (FAO) by directly activating PPARα, a key transcriptional factor regulating FAO [71], inducing carboxylesterase 1 (CES1) that mediates triglycerides hydrolysis and fuels mitochondrial FAO [72], and upregulating the expression and secretion of hepatic FGF21 [73], a fasting-induced hormone that promotes lipid oxidation and ketogenesis [74, 75]. Liver FXR activation was also shown to repress carbohydrate-response element-binding protein (ChREBP) [76] that regulates glycolysis-related genes and promotes hepatic lipogenesis under chronic hyperglycemia. Beyond regulating lipid metabolism, FXR also mediates glucose homeostasis. In the fed state, plasma glucose levels are lowered by FXR activation through repressed gluconeogenesis, improved glucose disposition and enhanced glycogen storage. Thus, $Fxr^{-/-}$ mice showed impaired glucose homeostasis, IR and diminished hepatic glycogen stores [77–79]. Bile acids affect the expression of gluconeogenesis-related genes, including phosphoenolpyruvate carboxykinase (PEPCK), glucose-6-phosphatase (G-6-Pase), and fructose-1,6-biphosphatase, which may be partially modulated by the inhibitory effects of SHP on HNF4α and FOXO1 [80, 81].

When it comes to the pathophysiological setting of obesity, the effect of FXR is less clear. Conflicting findings have unveiled the complexity of influence of FXR or SHP on lipid and glucose homeostasis. FXR activation improves diet-induced hepatic steatosis, hyperglycemia, and hyperlipidemia in diabetic mice [77, 82]. However, recent studies discovered that disturbance of hepatic FXR signaling reverses metabolic abnormalities. Glucose homeostasis and insulin sensitivity was demonstrated improved in *ob/ob* and HFD-fed mice under FXR deficiency, and long-term FXR activation aggravates HFD-induced weight gain and glucose intolerance by reducing energy expenditure [83–85]. Interestingly, unlike whole-body *Fxr*-null mice, these effects were not seen in liver-specific *Fxr* knockout mice, indicating intestinal FXR might exert a critical role in glucose metabolism. Besides, *Shp* deletion does not induce obesity, but IR occurs under the challenge of

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Western diet [86]. *Shp* deletion also prevents hepatic steatosis and protects against type 2 diabetes when combined with *Fxr* loss [87], implying coordinated roles of FXR and SHP on energy homeostasis. Moreover, the effect of TGR5 cannot be neglected. Disrupted hepatic FXR-SHP axis facilitates efficient fat usage in white adipose tissue and brown adipose tissue, which may be achieved by activating TGR5 in brown adipose tissue by abundant circulated bile acids in $Fxr^{-/-}/Shp^{-/-}$ mice [88]. In aged mice and obese mice induced by HFD, the combined loss of the hepatic FXR-SHP axis reverses the aging and metabolic dysregulation including increased body weight and adiposity, and glucose and insulin tolerance [87, 88], which maybe partially attribute to the involvement of diminished autophagic activity [89], and this phenotype is not improved in $Fxr^{-/-}$ or $Shp^{-/-}$ mice [88, 89]. These results together indicate that the mechanism of FXR- and SHP-mediated control of energy homeostasis is complexed and still needs more investigation.

The inter-organ regulation of intestinal FXR on liver lipid and glucose metabolism is also sophisticated. Steatosis-like symptoms such as elevated circulating plasma cholesterol and hepatic lipid levels including triglycerides and FFAs developed in *Fxr*-null mice can be ameliorated by recombinant FGF19 treatment [90], suggesting a possible role for intestinal FXR in hepatic lipid metabolism. Indeed, the intestine-selective FXR agonist fexaramine was demonstrated to promote adipose tissue browning by induction of FGF15 expression and reduction of obesity, steatosis and IR in mice [91]. However, it was later revealed that fexaramine treatment modifies the gut microbiota and changes the bile acid composition by increasing the fecal, intestinal, and serum levels of LCA, which has the strongest TGR5 agonism as an endogenous molecule that induces browning and improves insulin sensitivity by stimulation of GLP-1 release [92]. Intestinal FGF19 also favors the reduction of gluconeogenesis in the fed state via inhibition of cAMP regulatory element-binding protein (CREBP) and subsequent peroxisome proliferator-activated receptor-γ coactivator-1α (PGC-1α)-mediated PEPCK and G-6-Pase stimulation [93]. Most recently, a study using non-bile acid FXR agonist GSK2324 showed that FXR activation in mice or humans inhibits the expression of *Scd1*, diacylglycerol acyltransferase 2 (*Dgat2*), and *Lpin1* in a hepatic FXR-dependent manner, which specially represses hepatic lipogenesis, and limits intestinal lipid absorption due to the intestinal FXR-dependent reduction of bile acid pool size and changes in composition, highlighting the pivotal roles of both hepatic and intestinal FXR in NAFLD treatment [94]. Adding to the complexity, although FXR agonism has shown potential in the treatment of metabolic diseases in preclinical and clinical studies, *Fxr*-knockout mice are protected from obesity and IR, which leads to the hypothesis that absence of intestinal FXR might bring benefits to obesity-related disorders. T-βMCA levels are increased in germ-free mice or mice treated with antibiotic cocktail [95], which functions as an intestinal FXR antagonist and reduces FGF15 expression to increase hepatic bile acid synthesis and improve NAFLD and diabetes [34, 36, 96]. Other strategies targeting intestinal FXR including genetic knockout of FXR, gut bacteria modulation or treatment with a synthetic intestinal FXR-restricted antagonist glycine-β-MCA (GlyMCA) were shown to reduce intestine-derived and circulating ceramides by downregulation of intestinal ceramide synthesis-related genes [36, 97, 98]. Ceramides can cause endoplasmic reticulum (ER) stress and mitochondrial injury, and ultimately results in the activation of hepatic lipogenesis and gluconeogenesis in the pathogenesis of NAFLD [36, 96, 99]. Intriguingly, bile acid sequestration could also reverse liver injury and prevent progression of NASH by decreasing bile acid reabsorption, and thus inhibiting intestinal FXR and ceramide levels [100]. Furthermore, FXR in intestinal L cells diminishes proglucagon expression and GLP-1 secretion via interference with the glucose-responsive factor ChREBP, while

Fxr-null mice increases GLP-1 production and restores the balance of glucose metabolism [101].

The paradoxical roles of intestinal FXR in metabolic regulation are the subjects of debate (Fig. 4). Under obesity, intestinal FXR in humans and diet-induced obese mice is chronically activated, and a further increased intestinal FXR signaling by low-dose of FXR agonists results in elevated serum ceramide levels that potentiates metabolic disorders in intestinal *Fxr*-null mice [97, 98] and probably in humans, and therefore antagonism of FXR to prevent obesity-related diseases fits this scenario. However, the activated intestinal FXR signaling could also be considered as an adaptive mechanism to reduce fatty acid absorption under high fat treatment. Thus, the agonism of intestinal FXR exerts anti-obesity effects. Furthermore, most beneficial effects of intestinal FXR agonism rely on the interaction between FGF15/FGF19 and FGFR4/β-klotho pathway and the resulting modulation of bile acid synthesis, leading us to question whether there exists an "FGF15/19 resistance" in the pathogenesis of metabolic diseases. It remains to be determined the clinical relevance of intestinal FXR agonism and antagonism as well as the downstream effectors in the maintenance of hepatic lipid and glucose homeostasis.

Hepatic inflammation: FXR exerts an essential role on hepatic inflammation and fibrosis [102]. FXR activation can affect the innate immune system by activating liver natural killer T (NKT) cells. Induction of SHP by activation of FXR prevents the transcription activity of osteopontin induced by c-Jun and reduces osteopontin production, an extracellular matrix protein and immunoregulatory cytokine derived from NKT cells [103]. FXR activation can antagonize NF-κB-stimulated inflammation in liver through SUMOylation [104], increasing levels of NF-κB pathway inhibitory protein IκBα, the chaperone protein preventing nuclear translocation of p65 [105] or increasing the anti-inflammatory epoxyeicosatrienoic acids or reducing the production of inflammatory leukotrienes [106]. Apart from the canonical ligand-binding activation, FXR also physically interacts with NLR family pyrin domain containing 3 (NLRP3) and caspase 1 to repress inflammasome activation underlying cholestasis-associated liver inflammation [107].

Liver fibrosis: Hepatic inflammation drives HSCs activation, leading to liver fibrosis. Activation of FXR in HSCs aids in reducing hepatic fibrosis through different mechanisms. FXR activation induces the expression of SHP in HSCs and reduces HSC responsiveness to transforming growth factor beta (TGFβ) [108]. FXR activation can also reduce the expression of TGFβ and transforming growth factor beta receptor 2 (TGFβR2) [108, 109]. Moreover, FXR activation induces the expression of peroxisomal proliferator-activated receptor γ in HSCs, thereby reducing expression of inflammatory cytokines and collagen [110, 111]. FXR directly regulates the expression of perilipin-1 to stabilize lipid droplets and thereby prevents HSC activation [112]. These studies indicate that FXR could be a promising target for the treatment of NASH and fibrosis. However, in another study using mice and isolated HSCs, transactivation of FXR is gradually compromised during the process of HSC activation, which in turns renders HSCs insensitive to FXR agonists, and thus the combination of SUMOylation inhibitors and FXR agonists has superior anti-fibrotic efficacy as compared with FXR agonists alone [112]. It should also be noted that, different from the protective role of FGF15/19 in liver steatosis, FGF15/19 is not a direct profibrotic mediator or mitogen to HSCs, while FGF15 deficiency protects against liver fibrosis due to alternatively increased bile acid activation of FXR in HSCs [113].

Cell apoptosis and autophagy: Hepatocyte is vulnerable to apoptosis, which eventually contributes to many liver diseases including NASH [114]. It has been reported that FXR physically
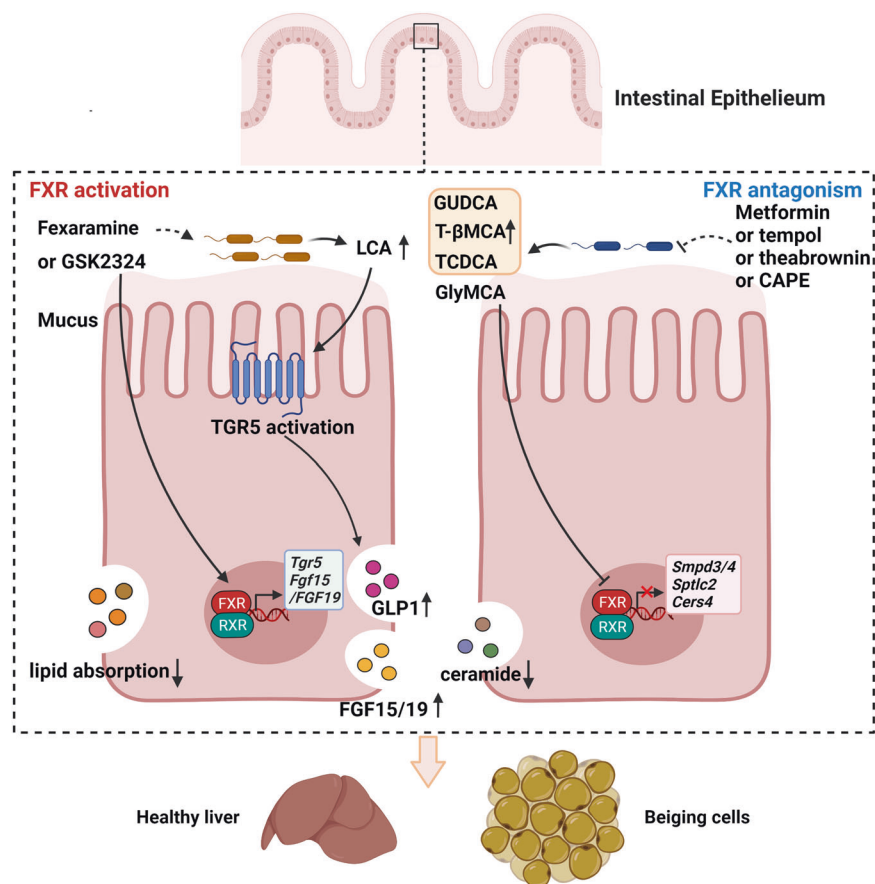
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Fig. 4 Paradoxical roles of intestinal FXR on the development of NASH. FXR agonist GSK2324 decreases hepatic triglyceride accumulation through a mechanism that lipid absorption decreased in an intestinal FXR-dependent manner. Intestine-specific FXR agonist fexaramine has a beneficial effect on glucose homeostasis in diet-induced obese mice by directly activating FXR, and alternatively increasing the abundance of lithocholic acid (LCA)-producing bacterium and circulating LCA levels, which thereby indirectly activates the TGR5-cAMP-GLP-1 cascade in intestinal L cells and induces the release of GLP-1 into the serum, eventually induces browning and improves insulin sensitivity. In addition, intestinal FXR activation induces the transcription of *Fgf15* (*FGF19* in humans) which is delivered to the liver and binds with FGFR4 to inhibit CYP7A1 expression and hepatic bile acid synthesis. FXR also activates TGR5 in enterocytes. Bile acid deconjugation can be decreased by metformin, tempol and theabrownin through reduction of the abundance of bile salt hydrolase (BSH)-secreting gut microbiota and BSH activity can be directly inhibited by caffeic acid phenethyl ester (CAPE), thus increasing levels of endogenous FXR antagonists glycoursodeoxycholic acid (GUDCA), taurochenodeoxycholic acid (TCDCA) and tauro-β-muricholic acid (T-βMCA), which reduced the ceremide synthesis-related genes including Smpd3/4, Sptlc2 and Cers4. As endogenous FXR antagonists are easily deconjugated by intestinal bacteria, synthetic glycine-β-muricholic acid (GlyMCA) was developed as a more stable FXR antagonist that benefited metabolic diseases via decreased ceramide synthesis. The figure was created with BioRender.com.

interacts with caspase 8, which thereby inhibits death receptors (DRs)-engaged apoptosis, ultimately attenuating both acute liver injury and chronic liver fibrosis in mice [115]. FXR-CYP4F axis also lowers intracellular 1-deoxysphingolipid levels, protecting hepatocytes from apoptosis [116]. Besides, by inhibiting bile acid synthesis, FXR activation prevents liver injury including apoptosis caused by accumulation of bile acids [117, 118].

Autophagy is the process of transporting intracellular material into subcellular structures called lysosomes, which are then degraded for recycling [119]. During periods of starvation, autophagy is activated to induce catabolic pathways that convert intracellular stores into energy to sustain survival [62]. Under fed state, FXR is activated to inhibit autophagy in the liver. Pharmacological activation of FXR inhibits autophagy in fasted mice, wheares FXR knockdown attenuates fed-mediated inhibition of autophagy. Mechanistically, activated FXR blocks autophagy by inhibiting the transcriptional activity of CREBP, which upregulates autophagic genes. Upon feeding or pharmacological activation, FXR mediates this inhibition by disrupting the functional interaction between CREBP and its coactivator protein CREBP regulated transcription coactivator 2 (CRTC2) [120]. It has also

been demonstrated that FXR binds directly to the promoter regions of genes that regulate autophagy, leading to their transcriptional suppression, and that PPARα could also bind to these sites. However, unlike FXR, PPARα is activated by fasting. PPARα induces some autophagic gene expression, which is inhibited by FXR. Thus, the two factors oppositely regulate the autophagic activity in response to nutrient supply by competing to bind a shared site on the autophagic gene promoter [121].

TGR5. The G protein-coupled bile acid receptor 1 (GPBAR1 or TGR5) is expressed in gallbladder epithelium, adipose tissue, and intestine, as well as hepatocytes. TGR5 is endogenously activated by bile acids with the rank order of potency LCA > DCA > CDCA > CA, and the taurine-conjugated bile acids generally more potent than the glycine-conjugated or unconjugated bile acids [14]. Interestingly, the microbiota-derived secondary bile acids LCA and DCA and their conjugates are the strongest activators of TGR5, thus inferring that the gut microbiota can potentiate bile acid signaling through TGR5.

Considerable studies have discovered the regulatory roles of TGR5 in lipid and glucose homeostasis, energy expenditure, and

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inflammatory response, which are tightly associated with NASH pathogenesis. TGR5 plays a key role in fasting-induced hepatic steatosis through modulation of CYP7B1 [122]. Activation of TGR5 can stimulate GLP-1 secretion from the ileal and colonic L-cells [123, 124], promoting insulin secretion of the pancreatic β cells and inhibiting the production of glucagon from the α cells. FXR and TGR5 are colocalized in enteroendocrine L-cells, and FXR activation can induce TGR5 [125]. Most recently, a study demonstrated a unique mechanism that HCA increases GLP-1 production and secretion in enteroendocrine cells via activating TGR5 and inhibiting FXR [126]. Moreover, activation of TGR5 in brown adipose tissue and skeletal muscle induces the expression of cAMP-dependent 2-iodothyronine de-iodinase (Dio2), converting the inactive thyroxine (T4) to active 3,5,3-triiodothyronine (T3), which is a major hormone in basal metabolism that causes more energy consumption and less body weight gain [127].

Activation of TGR5 also protects the liver from bile acid overload [128], markedly decreases TNFα and IL-12 in primary macrophages [129], as well as IL-1α, IL-1β, IL6 and TNFα in Kupffer cells via the TGR5-cAMP-dependent pathway [14]. LCA-activated TGR5 promotes the proliferation of cholangiocytes, following ERK1/2 phosphorylation [130]. Administration of 12α-OH bile acid such as TDCA and GDCA to mice significantly induces HSC proliferation and fibrosis through TGR5 mediated-p38 mitogen-activated protein kinase (MAPK) and ERK1/2 signaling pathway [53].

Vitamin D receptor. VDR is a member of nuclear receptor superfamily. The canonical ligand of VDR is vitamin D [131]. Under cholestasis, VDR is activated by accumulated LCA, thereby inhibiting bile acid synthesis [132]. LCA also induces CYP3A4 expression in the liver, mediating bile acid oxidation and cell detoxification through VDR activation [133]. *Vdr*-null mice spontaneously develops hepatic fibrosis, and VDR activation in HSCs can improve fibrogenesis probably via negative regulation of p62 [134]. The hepatic steatosis is improved in a global *Vdr*-deficient mouse model, indicating the value of studying the role of VDR in NASH [135]. However, the liver becomes more vulnerable to steatosis with a HFD by disruption of hepatic VDR [136], implying different functions of hepatic and intestinal VDR. It was reported that *VDR* mRNA is increased in NAFL patients, and this increase is positively correlated with the expression of angiopoietin-like protein 8 (ANGPTL8) and steatosis grade [137], whereas knockdown of ANGPTL8 dampens FFA-induced triglyceride accumulation. Thus, the relationship between VDR and hepatic steatosis needs further investigation.

PPARα. The ligand-activated transcription factor PPARα is well-known for coordinating different metabolic pathways in liver under fasting status [75, 138, 139]. Currently, the role of PPARα on the regulation of bile acid homeostasis is emerging. CA and CDCA were reported to have an antagonistic effect on murine PPARα [140]. An earlier study found that clofibrate activates PPARα and reduces bile acid synthesis by affecting the expression of *Cyp7b1*, *Ntcp*, *Oatp4* and *Bsep* [141, 142]. What's more, PPARα plays a main anti-inflammatory role in human liver [143]. A recent study showed that PPARα activation attenuates NLRP3 inflammasome activation, caspase-1 cleavage, and proinflammatory IL1β maturation by upregulating the long non-coding RNA gene Gm15441 [144]. Loss of hepatic PPARα promotes inflammation and serum hyperlipidemia in HFD-induced obesity [145]. PPARα selective agonist pemafibrate was demonstrated to prevent NASH development but not alter hepatic triglyceride accumulation by decreasing recruitment of myeloid cells, which is via interaction with liver sinusoidal endothelial cells [146]. The above findings make PPARα an attractive target for the treatment of NASH.

TRβ. Most recently, hypothyroidism has been considered as an influential factor in NAFLD, independent of thyroid hormone (TH) level [147]. TH regulates cellular and tissue metabolism by binding to thyroid hormone receptors (TRs). To date, two TR isomers, TRα and TRβ, have been identified, with TRβ abundant in the liver [148]. It has been reported that activation of hepatic TRβ is associated with increased bile acid synthesis, as shown by effects of TH on cholesterol metabolic genes [149, 150]. It has also been shown that TH induces bile acid synthesis in humans [151]. TH is demonstrated to regulate SHP mRNA through interference with the transcription factor LRH-1 [152], and *Cyp7a1* is a direct TR target gene that responds to physiologic TR levels through a set of distinct response elements in its promoter [150]. Apart from the effect on bile acid synthesis, TRβ plays a pivotal role in maintaining liver lipid homeostasis. Activation of hepatic TRβ is associated with reduced systemic lipids and increased lipid oxidation [153], and also improves disease phenotype without body weight gain [154]. The expression of TRβ in the liver is reduced in NASH [155], rendering it a potential target for NASH treatment. Accumulated data have shown that TRβ agonists in clinical development have metabolic benefits in both preclinical models of NASH animals and patients, including reduced fibrosis, as well as improved quality of life [154, 156, 157], which may be attributed to the induction of genes related to cholesterol and fatty acid biosynthesis and metabolism such as carnitine palmitoyltransferase 1 A (*Cpt1a*) and angiopoietin-like protein 4 (*Angptl4*) [158]. The bile acids increased by TRβ agonists may cause subsequent hepatic injury, where synergistically beneficial effects may be realized by combination of TRβ agonists with other therapeutic modalities, such as FXR agonists.

Bile acids and inflammation
In addition to their critical roles on regulating lipid and glucose homeostasis, bile acids are recognized as important molecules on immune regulation. The role of bile acids on inflammatory liver diseases such as cholestasis has been extensively studied [159, 160], while in NASH, which is now characterized as a metabolic factors-mediated liver dysfunction, these immune regulatory activities are mainly achieved by two bile acid receptors including FXR and TGR5, which are highly expressed in cells of innate immunity. Currently, accumulated evidences have disclosed the possible role of gut dysbiosis in NASH [161, 162]. Since bile acids are transformed by gut microbes and can shape the pattern of gut microbiota in the intestine [163, 164], it is rational that there is a reciprocal effect of gut microbes and bile acids in the development of NASH. As key regulators of intestinal inflammation, the effect of gut microbes and their derived metabolites including short-chain fatty acids, choline and choline-related metabolites, ethanol, as well as bile acids on NASH progression should be put more emphasis. More simply, bile acids could also initiate immune response through their detergent features leading to cellular apoptosis [115, 118, 165, 166].

Progress in drug discovery
Bile acid analog. UDCA, also known as ursodiol, is a naturally occurring bile acid used as a first-line drug in the treatment of primary biliary cholangitis [167]. In addition to the cholestatic liver diseases, UDCA was found effective in protecting hepatocytes from oxidative damage [168] and reducing alanine transaminase (ALT) levels in NASH patients [169], as well as attenuating liver inflammation and fibrosis by reducing oxidative stress in a diet-induced NASH model [170]. Conflicting data were observed in other cohorts, as UDCA at a dose of 13–15 mg·kg^{-1}·d^{-1} was not efficacious in patients with NASH [171], while high doses of UDCA (23–28 mg·kg^{-1}·d^{-1}) did not improve overall liver histology compared with placebo over an 18-month treatment period [172]. Moreover, the mechanism by which UDCA reduces primary biliary cirrhosis and hepatic bile acids remains elusive, since UDCA has no direct effect on neither FXR nor TGR5 signaling. When UDCA enters the liver, it is extensively converted to its conjugated forms TUDCA and GUDCA, which were demonstrated to be FXR antagonists, and TUDCA is cytoprotective as a potent inhibitor of

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ER stress which is one of determinant factors in NASH [173]. Therefore, the therapeutic effect of UDCA on NASH still needs to be further addressed.

24-Norursodeoxycholic acid (NorUDCA), a derivative of UDCA, is relatively resistant to amidation. Therefore, the hepatic elimination of NorUDCA is lower than UDCA, enabling the enrichment of NorUDCA in liver [174, 175]. Additionally, NorUDCA treatment demonstrated a dose-dependent reduction in serum ALT at a dose of 500 mg·d^{-1} or 1500 mg·d^{-1} when compared with placebo [176].

Arachidyl-amido cholanoic acid (Aramchol) is a novel fatty acid-bile acid conjugate that targets SCD1 in HSCs [177], the enzyme responsible for monounsaturated fatty acids synthesis. In a phase IIb clinical trial, Aramchol administered to patients with NAFLD for 1 year was shown to improve fibrosis without worsening NASH (NCT02279524), making it a promising candidate for NASH therapy. Currently, a phase III clinical trial for NASH patients is ongoing (NCT04104321). Aramchol meglumine (salt compound of Aramchol) is an improved compound that increases the solubility and is effective in increasing the concentration of Aramchol in plasma. To facilitate its widespread use in other oral formulations, Galmed Pharmaceutics Ltd. plans to apply Aramchol meglumine in a phase III clinical trial (ARMOR). Amilo-5MER, a 5 amino acid synthetic peptide is also being developed and recently initiated a first-in-human study.

FXR agonist. A number of FXR agonists are currently undergoing clinical evaluation, and could be structurally divided into either bile acid analogs or non-steroidal agonists. Obeticholic acid (OCA, INT-747) is a selective FXR agonist that is structurally derivative of CDCA with higher potency (up to 100-fold) than CDCA [178]. In patients, it has been shown to improve liver function of NASH patients [179]. In the phase II study (FLINT), all histological hallmarks of NASH and fibrosis grade were improved in the OCA groups [179]. Data from REGENERATE showed that patients had improved liver fibrosis beyond grade 1 during 18 months of OCA treatment (18% and 23% with OCA 10 and 25 mg versus 12% placebo), and a higher percentage of NASH patients in the OCA-treated group had no worsening symptoms compared to the placebo group [180]. In a study of diabetic NAFLD patients orally dosed with 25 mg OCA for 6 weeks, total cholesterol levels were not increased but high-density lipoprotein (HDL) levels did fall [181]. However, there are severe side effects such as dose-dependent pruritus and low-density lipoprotein (LDL) elevation [179], which might result from its strong activity and low selectivity thus limiting the clinical use of OCA. Especially, the increase of LDL-cholesterol requires a lipid-lowering therapy with statins in half of the patients in the OCA group (REGENERATE). On November 25, 2019, Intercept announced that the U.S. Food and Drug Administration (FDA) accepted a New Drug Application submission of OCA for the treatment of NASH with fibrosis and granted priority review [182]. However, on June 29, 2020, Intercept announced that the marketing application for OCA for the treatment of NASH-induced liver fibrosis was denied by the FDA. A second phase III study (REVERSE) of OCA in NASH patients with compensated cirrhosis is currently ongoing, which will answer the question that whether OCA therapy is safe and effective in this advanced stage of the disease.

The need to avoid the side effects associated with the steroidal structure of OCA led to the discovery of non-steroidal FXR agonists because of their better water solubility, as well as their better efficacy and safety. Tropifexor (NVP-LJN452), a non-steroidal, non-bile acid FXR agonist, showed better efficacy compared to OCA in different preclinical NASH models [183, 184]. In healthy volunteers, tropifexor showed a good safety and tolerability profile [185]. In a phase II clinical trial, tropifexor was able to reduce hepatic lipids, ALT and gamma-glutamyl transferase in patients (NCT02855164). GS-9674 (Cilofexor, Gilead Sciences) is another FXR agonist that significantly reduces hepatic steatosis, serum bile acids and hepatic biochemical parameters without altering serum lipids. In a phase II RCT, NASH patients treated with GS-9674 (100 mg) had a decreased liver fat content by 22.7% ($P = 0.003$) after 24 weeks compared to placebo, with 39% of patients experiencing a 30% decrease in liver fat content ($P = 0.011$) [186]. The PX-104 is another oral non-steroidal agonist of FXR and it can improve insulin sensitivity and liver enzymes in non-diabetic NAFLD patients [187], and the clinical application to NASH patients needs further preclinical investigation. MET409, a new non-bile acid agonist with a novel chemical structure, significantly reduced liver fat and attenuated NASH after 12 weeks of treatment (50 or 80 mg·d^{-1}) [188]. Other novel FXR agonists, such as nidufexor (LMB763), EDP-305 and TERN-101 are currently being tested in clinical studies for the treatment of NASH [18].

Unexpectedly, similar to OCA, pruritus and changes in serum cholesterol pool with decreased HDL and increased LDL were also observed during treatment with non-bile acid FXR agonists, despite their non-steroidal nature, suggesting potential on-target side effects [189, 190]. More studies should be conducted to examine the safety and efficacy of FXR agonists in combination with another complementary drug aiming to lower dosage and side effects, such as statins. The class-specific side effects might be associated with systemic exposure of FXR agonists. To overcome these limitations, the strategy that targets intestinal FXR was suggested. Some studies have shown the beneficial metabolic effects of intestine-restricted FXR agonists fexaramine and Fex-3 in diet-induced obese mouse models [91, 191].

FXR antagonist. It was shown that intestinal FXR is a promising target for the treatment of obesity and NAFLD [34, 35]. The natural FXR antagonist T-βMCA in mice and GUDCA in humans is rapidly hydrolyzed by BSHs, which reduces their inhibitory effect, resulting in the seeking of small molecule FXR inhibitors. GlyMCA, which is a derivative of T-βMCA and resistant to BSH hydrolysis, is selectively accumulated in gut, not liver, and then ameliorates diet-induced obesity, NAFLD, and diabetes in mouse models [97, 192, 193]. Other alternative strategies to enrich nature bile acid antagonists in gut are discovered for the treatment of NAFLD. Tempol, metformin and theabrownin could reduce the abundance of BSH-secreting bacteria, and caffeic acid phenethyl ester could directly inhibit BSH activity. This would decrease the hydrolysis of bile acids and increase the level of endogenous FXR antagonists [36, 37, 194]. However, this antagonist should act only in the intestinal tract, otherwise it can cause other side effects, such as inhibition of hepatic FXR, leading to cholestasis and HCC [195, 196].

TGR5 agonist. INT767, a steroidal FXR/TGR5 dual agonist, promotes fat uptake by adipocytes via activating FXR and enhances GLP-1 secretion by activating TGR5. INT767 normalizes cholesterol and triglyceride levels [197] in diabetic mice and reduces hepatic steatosis, and shifts monocytes and macrophages to an anti-inflammatory M2 phenotype [198]. In a 16-week NASH rat model, INT767 treatment improved lipid and glucose metabolism, reduced IR and attenuated the pro-inflammatory response [199]. Furthermore, INT767 exerts greater therapeutic potency and efficacy than OCA in the *ob/ob* NASH mouse model [200]. INT767 is now in a phase I clinical trial. BAR502, a nonsteroidal dual FXR and TGR5 agonist derived from UDCA, regulates NKT cells during hepatitis in mice [201–203]. However, systemic TGR5 activation have until now been linked to some undesirable side effects, including induction of gallstone formation, and obstruction of gallbladder emptying [204, 205]. An intestine-restricted TGR5 agonist, RDX8940, was found to induce incretin secretion, improve hepatic steatosis without inhibiting gallbladder emptying in a mouse model of NASH [206].

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Other therapies related to bile acid metabolism. Bile acid sequestrants bind bile acids in the intestine and their complexes with bile acids are non-absorbable and not easily destroyed. As a result, bile acid sequestration induces feedback regulation of bile acid synthesis by inhibiting intestinal FXR-FGF15 signaling and allowing the conversion of cholesterol to bile acids by upregulating CYP7A1, which in turn leads to a decrease in total plasma cholesterol and LDL-cholesterol levels [100]. Sevelamer is a novel phosphate binding agent for the treatment of hyperphosphatasia in chronic kidney disease [207]. It was shown to prevent the NASH phenotype, associated with increased *Lactobacillus* and reduced *Desulfovibrio*, thus coordinating inflammation response, and glucose and lipid metabolism [100].

Aldafermin (NGM282), a bioengineered nontumorigenic FGF19 analog, can inhibit *de novo* lipogenesis, improve insulin sensitivity and correct mitochondrial dysfunction [18]. In a 24-week phase II clinical trial (NCT02443116), NGM282 can produce a trend toward fibrosis improvement in NASH [208]. At week 12 in this trial, NGM282 produced rapid and significant reduction in liver fat content in NASH patients [209]. It is worthy of note that NGM282 produced significantly greater decrease in levels of C4 and total bile acids in histological responders than in non-responders [209]. Up to now, the upcoming phase III RCT is worthy of being reported.

More recently, intestine-specific ASBT inhibitors have been suggested to have potential in the treatment of NASH, such as increasing GLP-1 secretion, lowering plasma glucose and decreasing plasma cholesterol [210–212]. In addition, since ASBT inhibitors reduce the circulation of bile acids to the liver, they can be applied in cases of impaired liver function, but they can also cause adverse effects such as diarrhea. The therapeutic mechanism of ASBT inhibitors in NASH treatment needs further clarification.

Traditional Chinese medicine has a wide range of clinical use in Asian countries, with a long and profound history. Berberine is an isoquinoline alkaloid isolated from the rhizome of the perennial herb *Coptis chinensis*. Its therapeutic effects on NAFLD and diabetes were demonstrated [213, 214], and it has produced a significant reduction in hepatic lipid accumulation, inflammation and liver fibrosis [215]. Currently, there are also herbal formulas that were shown to be effective in the treatment of NAFLD [216, 217]. Beyond the physically mixed formulations, a new molecular entity, HTD1801 (berberine ursodeoxycholate or BUDCA), is designed as an ionic salt formed between berberine and UDCA. An 18-week phase II study (NCT03656744) showed that HTD1801 controlled blood glucose and reduced liver fat content, liver enzymes and body weight [218]. The most frequently reported adverse events for HTD1801 are diarrhea and abdominal discomfort, which are commonly encountered for both UDCA and berberine. Studies of HTD1801 in biopsy-confirmed NASH patients are expected to investigate its efficacy on the more severe fatty liver disease. Furthermore, it should also be answered that whether this ionic salt form of berberine and UDCA induces a better response in patients when compared to single drug.

Recently, probiotics or nondigestible prebiotics have been suggested to provide benefits for metabolic diseases by modifying the gut microbiota. VSL#3, a mixture of several probiotic bacteria with active BSH activity, induces bile acid deconjugation and increases bile acid synthesis, and thus improves insulin sensitivity and protected against NASH in mice [219, 220]. Prebiotics may have a beneficial effect on NAFLD, potentially via modulation of bile acid metabolism. However, the role and safety profile of these live probiotics still need to be studied.

PPAR agonists including saroglitazar and lanifibranor (IVA337), and TRβ agonists including resmetirom and VK2809 for NASH treatment are in clinical development [18]. Currently, the therapeutic actions of these agonists on NASH mainly benefit from their lipid-lowering effect to reduce hepatic fat content. The influence of these agonists on bile acid dysregulation in NASH pathogenesis are required further investigation.

Combination therapeutic strategies for NASH. Given the heterogeneous pathogenesis of NASH, it is now recognized that multiple mechanistic pathways should be concurrently targeted to achieve the overall metabolic benefits and obtain an optimal treatment response. Several combinatory therapies are currently developed and most of them including an FXR agonist. A phase II study (ATLAS) compared the safety and efficacy of using cilofexor in single- or dual-drug combinations with the ACC1 inhibitor firsocostat or the apoptosis signal-regulated kinase 1 (ASK1) inhibitor selonsertib for the treatment of advanced fibrosis. After 48 weeks of treatment, a grade one improvement in fibrosis was found in the combination treatment group (sirofosfamide and fosfestrol), while there was no worsening of NASH [18]. Another phase II study in advanced NASH patients has shown that combination regimens including a GLP-1 receptor agonist semagalutide with cilofexor and firsocostat led to greater improvements in hepatic steatosis, despite similar reductions in body weight as semaglutide alone. Other clinical trials are currently underway to test the combination of tropifexor with a sodium glucose co-transporter 1/2 (SGLT1/2) inhibitor licogliflozin or with the leukotriene A4 hydrolase inhibitor LYS006 [18].

CONCLUSION AND PERSPECTIVE

Bile acids can be both physiological detergents and key metabolic regulators, mediating metabolic homeostasis and inflammation. The disturbance of bile acid pool has been reported in NASH patients, making bile acid a potential biomarker for NASH. Currently, the bile acid receptors are considered strong targets for the treatment of NASH (Table 2), considering its key role in pathophysiological processes involving steatosis, inflammation or fibrosis. Among all the drug targets for NASH, FXR is the one most widely studied and several structurally-different FXR agonists are currently under development. According to their published results [179, 180, 182, 185–187], OCA has become the most promising, which is undergoing a second phase III study (REVERSE) in NASH patients with compensated cirrhosis. However, either steroidal OCA or non-steroidal agonists all suffered from dose-dependent side effects, including pruritus and increased plasma cholesterol and LDL, along with reduced HDL, suggesting the association with systemic FXR activation. To overcome the unwanted side effects, intestinal restricted FXR agonists might be better tolerated. But there is another known risk related to the highly-induced tumorigenic FGF19 circulating to liver. Apart from agonists of FXR, antagonists of intestinal FXR have attracted much attention, which provides a new insight for different functions of bile acid receptors in inter-organ communication. Alternatively, the combinatory use of FXR agonist with lipid-lowering agents such as statins will also help lower the dosage of FXR agonist and minimize side effects. The balance between efficacy and safety needs to be carefully evaluated.

Based on the latest progression on clinical trials, it seems like a challenging goal to tackle with all the three manifestations of NASH interfering with one single active molecule, since the pathogenesis of NASH is complex. Whether the combination of molecules with different modes of action will bring better benefits still needs further evaluation, particularly in NASH patients with other complications. Moreover, the differences in bile acid composition between humans and animals, for example, MCA produced by mice are absent in humans, have possibly led to high rates of clinical failure, raising the requirements of the development of better animal models or earlier evaluation in primates.

Finally, NASH is a heterogeneous disease with complex systemic metabolic abnormalities, rather than a single liver disorder in lipid accumulation. The initial signal, such as lipid accumulation, IR, and lipotoxicity are relatively well established, while the downstream processes, such as hepatocyte apoptosis, infiltration of

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Table 2. List of anti-NASH drugs

Name of drug	Mechanism of action	Effects	Phase in clinical trial	Clinical manifestations
UDCA	Bile acid analog	Showed antioxidation, anti-inflammation and anti-fibrosis potency	——	1. Ineffective after treatment with a dose of 13–15 mg·kg⁻¹·d⁻¹ 2. Failed to improve overall liver histology after treatment with 23–28 mg·kg⁻¹·d⁻¹ for 18 months
NorUDCA	Bile acid analog	1. Previously used in the treatment of primary biliary cholangitis 2. Reduced liver steatosis, inflammation and fibrosis	——	Showed a dose-dependent decrease in serum ALT of patients with NAFLD after 12 weeks of treatment with doses of 500 mg·d⁻¹ or 1500 mg·d⁻¹
Aramchol	Bile acid analog	Inhibited lipogenesis and improved fibrosis	III	Showed an improvement in fibrosis without worsening NASH after treatment of NAFLD patients for 1 year
HTD1801	Bile acid analog	Decreased lipid accumulation, inflammation and fibrosis in the liver	II	1. Improved blood sugar, reduced hepatic fat content and body weight 2. Adverse effects: diarrhea and abdominal discomfort
OCA	FXR agonist	Diminished liver steatosis, inflammation and fibrosis and improved insulin sensitivity	III	1. Improved fibrosis in NASH patients 2. Adverse effects: pruritus and elevated LDL
Tropifexor (NVP-LJN452)	FXR agonist	Improved cholestatic liver injury and fibrosis	II	Reduced liver lipids, ALT and gamma-glutamyl transferase
GS-9674	FXR agonist	Reduced hepatic triglyceride accumulation	II	Showed a significant reduction in liver fat content of NASH patients after 24-week treatment with GS-9674 (100 mg)
PX-104	FXR agonist	Improved insulin resistance	II	Improved insulin sensitivity of non-diabetic NAFLD patients after 4-week treatment
MET409	FXR agonist	Reduced liver fat content	II	1. Reduced liver fat content 2. Adverse effects: pruritus and elevated LDL-cholesterol levels
GlyMCA	FXR antagonist	Improved diet-induced obesity-related metabolic dysfunctions in mouse models	——	——
Metformin	FXR antagonist	1. Interacted with intestinal flora and reduced the abundance of BSH-secreting bacteria 2. Inhibited hepatic glucose synthesis and increased insulin sensitivity	——	1. Reduced body weight and improved insulin resistance 2. Ineffective in improving liver histology, including steatosis and inflammation 3. Mostly used for NAFLD patients with prediabetes or concomitant diabetes
INT767	FXR/TGR5 agonist	1. Reduced liver steatosis, inflammation and improved insulin resistance 2. Adverse effects: obstruction of gallbladder emptying and induction of gallstone formation	——	——
BAR502	FXR/TGR5 agonist	1. Promoted white adipose tissue browning and reversed diet-induced hepatic steatosis and fibrosis 2. Adverse effects: obstruction of gallbladder emptying and induction of gallstone formation	——	——
RDX8940	TGR5 agonist	Induced intestinal insulin secretion, improved hepatic steatosis and insulin sensitivity	——	——
Sevelamer	Bile acid sequestrant	1. Previously used to treat hyperphosphatasia in chronic kidney disease 2. Coordinated inflammatory response, and glucose and lipid metabolism	——	——
NGM282	FGF19 analog	1. Inhibited lipogenesis, improved insulin sensitivity and reduced inflammation and fibrosis 2. Significantly reduced the level of 7α-hydroxy-4-cholestene-3-one and total bile acids	II	Significantly reduced hepatic lipid accumulation and had a tendency to improve NASH fibrosis
Saroglitazar	PPAR-α/γ agonist	1. The first approved drug for the treatment of NASH 2. Reduced plasma lipid levels and improved insulin sensitivity	III	Achieved primary and secondary clinical endpoints in the phase III clinical trial
Lanifibranor (IVA337)	PPAR-α/δ/γ agonist	Diminished liver fibrosis and inflammation	III	Achieved primary and secondary clinical endpoints in the phase IIb clinical trial
Resmetirom (MGL-3196)	TRβ agonist	Decreased liver fat content	III	Reduced at least 50% of liver fat from baseline in more than 50% of patients at week 16 of the phase III clinical trial
VK2809	TRβ agonist	Reduced liver fat content and lowered high blood pressure	II	Significantly lowered liver fat, body mass index and hypertension

proinflammatory cells, as well as hepatic stellate cell activation and fibrogenesis are incompletely understood. Therefore, multi-targeted therapy is suggested, especially combining FXR agonists with lipid regulators, insulin, insulin sensitizers or GLP-1 in lipid and glucose metabolism, CC chemokine receptor 2/5 (CCR2/CCR5) dual agonist in inflammatory response, and ASK1 inhibitor in fibrogenesis. Classification of patient subsets based on biomarker screening is also needed.

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