Bile acids and sphingolipids in non-alcoholic fatty liver disease

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Abstract
Non-alcoholic fatty liver disease (NAFLD) is one of the fastest-growing diseases, and its global prevalence is estimated to increase >50% by 2030. NAFLD is comorbid with metabolic syndrome, obesity, type 2 diabetes, and insulin resistance. Despite extensive research efforts, there are no pharmacologic or biological therapeutics for the treatment of NAFLD. Bile acids and sphingolipids are well-characterized signaling molecules. Over the last few decades, researchers have uncovered potential mechanisms by which bile acids and sphingolipids regulate hepatic lipid metabolism. Dysregulation of bile acid and sphingolipid metabolism has been linked to steatosis, inflammation, and fibrosis in patients with NAFLD. This clinical observation has been recapitulated in animal models, which are well-accepted by experts in the hepatology field. Recent transcriptomic and lipidomic studies also show that sphingolipids are important players in the pathogenesis of NAFLD. Moreover, the identification of bile acids as activators of sphingolipid-mediated signaling pathways established a novel theory for bile acid and sphingolipid biology. In this review, we summarize the recent advances in the understanding of bile acid and sphingolipid-mediated signaling pathways as potential contributors to NAFLD. A better understanding of the pathologic effects mediated by bile acids and sphingolipids will facilitate the development of new diagnostic and therapeutic strategies for NAFLD.

Keywords: Bile acids; Sphingolipids; Non-alcoholic fatty liver disease; Non-alcoholic steatohepatitis

Introduction
Non-alcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver disease worldwide, with 28 to 52 new cases per 1000 individuals diagnosed annually.³⁴ NAFLD’s prevalence is the highest in the Middle East (31.90%), followed by South America (30.45%), Asia (27.37%), and the United States (24.13%).³⁴ Non-alcoholic fatty liver (NAFL) is a form of NAFLD with simple hepatic steatosis and little or no inflammation.³⁴ Non-alcoholic steatohepatitis (NASH) is the advanced form of NAFLD with inflammation and varying degrees of liver damage. The majority of NAFLD patients have NAFL, and only 10% to 25% of NAFLD patients develop NASH. Patients with NASH have an increased chance of developing end-stage liver disease and hepatocellular carcinoma (HCC).³⁵ In 2017, a Global Burden of Disease study conducted by the Institute of Health Metrics and Evaluation showed that 3.1% and 5.6% of liver-related deaths were due to NAFLD-associated HCC and cirrhosis, respectively.⁶ NAFLD also increases the risk and incidence of extrahepatic cancers. In 2020, clinical assessment of patient data in a retrospective cohort study of 254 patients with bladder cancer or disease between late-middle and advanced age groups (61–77 years) in Italy showed that high serum triglycerides (TGs) were significantly associated with non-muscle-invasive bladder cancer (NMBC) incidence.⁷ Although NMBC-NAFLD did not meet statistical significance (P=0.06), its near-significant positive association suggests its biological relevance. In 2021, a meta-analysis comprising data from over 182,000 middle to late-middle-aged individuals was conducted to assess cancer incidence among patients with NAFLD over 6 years, including 45,000 patients with NAFLD, and demonstrates that NAFLD diagnosis increases the risk of developing extrahepatic cancers of the gastrointestinal tract, lung, reproductive system, or urinary tract.⁸ Collectively, these data show that NAFLD can have a broad and significant clinical impact on patient health. Furthermore, the inclusion of NAFLD diagnosis as a risk factor for cancer development may encourage more regular cancer screenings and improve patient outcomes.
across multiple medical specialties by detecting early tumor establishment.

In the past few decades, extensive studies have shown that NAFLD is a metabolic disease closely associated with obesity, type 2 diabetes, lipid metabolism, hepatic bile acid dysregulation, and, especially, insulin resistance.[10,11] Recently, a group of experts recommended renaming “NAFLD” to “MAFLD,” metabolic-associated fatty liver disease, to more accurately reflect pathogenesis and improve the clinical management of NAFLD.[12-14] Although extensive efforts have been put forth to develop pharmacotherapies, no FDA-approved pharmacologic or biologic interventions exist for NAFLD due to the incomplete understanding of the complicated pathogenesis from NAFL to NASH.[15] As a result, dietary modifications are the current gold standard for the modulation of NAFLD progression.[16] In response to the apparent clinical need for NAFLD treatment options, research into the cellular mechanisms which underlie NAFLD is essential for developing novel targeted therapeutics.

It has been well recognized that bile acids are essential signaling molecules and play critical roles in regulating hepatic lipid metabolism. Identification of the cross-talk between bile acid and sphingolipid-mediated signaling pathways opened a new direction for studying bile acids and sphingolipids in hepatic lipid metabolism and provided new opportunities to develop potential diagnostic and therapeutic tools for NAFLD and other metabolic diseases.[17-20] Sphingolipids are one of the oldest discovered lipids and have been extensively studied as essential structural components of lipid rafts and important signaling molecules, which play significant roles in regulating various physiological functions.[21] In the past decade, numerous studies have shown that sphingolipid metabolism dysregulation is closely associated with multiple metabolic disorders, including NAFLD.[17-20]

Furthermore, the role of bile acid-mediated signaling pathways in NAFLD has recently been extensively discussed.[22,23] In this review, we will highlight the recent advances in the sphingolipid field and discuss connections between bile acids and sphingolipid signaling with a focus on NAFLD.

Pathology of NAFLD

NAFLD is defined as a type of fatty liver disease not caused by alcohol consumption. There are two forms of NAFLD: NAFL and NASH. NAFL is characterized by simple fat accumulation with at least 5% steatosis but little or no inflammation and hepatocellular damage.[24] NASH is a more active form of NAFLD characterized by significant inflammation, ballooning, and fibrosis, which can progress to advanced fibrosis, cirrhosis, and HCC [Figure 1].[24] The global incidence and prevalence of NAFLD are rapidly increasing along with the obesity pandemic. It is estimated that the global NASH prevalence will increase >50% by 2030. In addition, the incidence of NASH-related HCC is projected to increase significantly and become the fastest-growing cause of liver transplantation in the United States.[25]

In the past several decades, extensive effort has been put into identifying potential mechanisms underlying NAFLD pathogenesis. It is well accepted that NAFLD is closely associated with obesity, insulin resistance, gut dysbiosis, inflammation, and disruption of bile acid and lipid metabolism.[26] In addition, genome-wide association studies have identified genetic risk mutants related to NAFLD, such as PNPLA3, TM6SF2, and SREPI.[27] Both clinical and pre-clinical studies indicate that dysregulation of sphingolipid metabolism is closely associated with NAFLD disease progression.[18,21] However, neither FDA-approved pharmacotherapies for...
NASH nor biomarkers for diagnosis or assessment of NASH disease progression and therapeutic outcomes are available.\[28\]

Bile Acid and Sphingolipid Metabolism

Bile acid metabolism and signaling

Bile acids are synthesized from free cholesterol in the hepatocytes via two well-characterized pathways, the classical or neutral pathway and the alternative or acidic pathway. The classical/neutral pathway produces ≥90% bile acid, while the alternative/acidic pathway produces the remaining ≤10%. Cholesterol 7α-hydroxylase (CYP7A1) and cholesterol 27α-hydroxylase (CYP27A1) are the rate-limiting enzymes for the classical pathway and alternative pathway, respectively. CYP7A1 is only expressed in hepatocytes, but CYP27A1 is expressed in mitochondria in multiple cell types and tissues.\[23,29\]

Cholic acid (CA) and chenodeoxycholic acid (CDCA) are two primary bile acids in humans. In mice, CDCA is further converted into α-muricholic acid (α-MCA) via 6β-hydroxylation by a cytochrome P450 enzyme, Cyp2c70. In humans, CA and CDCA are conjugated with glycine or taurine by bile acyl-coenzyme A (CoA) synthetase and bile acid-CoA: amino acid N-acyltransferase to form conjugated bile acids (CBAs).\[23\] These bile salts aggregate as micelles and mix with water, cholesterol, bilirubin, and trace minerals and are stored in the gallbladder as bile.\[30,31\] Under normal physiological conditions, bile acids undergo enterohepatic circulation three to five times per day. Enterohepatic circulation involves the secretion of bile acids from the gallbladder to the intestinal lumen, where bile acids facilitate lipid-soluble nutrient absorption. More than 95% of bile acids are reabsorbed by enterocytes in the terminal ileum and transported to the liver via the portal vein.\[23,32\] About 5% of bile acids escape reabsorption and will be transformed into a variety of secondary bile acids by gut bacteria via deconjugation, oxidation, esterification, epimerization, and desulfation. CA and CDCA are transformed into deoxycholic acid and lithocholic acid (LCA) in humans. CDCA also can be transformed into ursodeoxycholic acid. In mice, α-MCA and β-MCA are converted into murideoxycholic acid. In addition, β-MCA also can be transformed into ω-MCA, hyocholic acid, and hyodeoxycholic acid. The 5% of bile acids excreted in feces are replaced through de-novo synthesis in the liver\[23\] (Figure 2).

Bile acids are not only detergents but are also important signaling molecules. In the past 2 decades, numerous studies have shown that bile acid-mediated activation of the nuclear receptor, farnesoid X receptor (FXR), and G protein-coupled receptors (GPCRs), Takeda G-protein-
coupled receptor 5 (TGR5) and sphingosine-1 phosphate receptor 2 (S1PR2), play important roles in the progression of NAFLD and other metabolic diseases via modulating hepatic lipid metabolism, immune responses, and the gut microbiome.[22,23,33]

**Sphingolipid metabolism and signaling pathways**

Sphingolipids are essential structural components of all eukaryotic membranes. The synthetic pathways of sphingolipids have been well characterized. More than 40 enzymes are involved in de-novo synthesis, catabolism, recycling, and interconversion of these lipids in reversible reactions.[21] Ceramide is the core molecule of the complex sphingolipids. In canonical biosynthesis, ceramide is predominately produced in the endoplasmic reticulum (ER). The initial step of the de-novo synthesis of ceramide occurs through the condensation of palmitoyl-CoA and L-serine to form 3-keto-dihydro sphinganine (3-KDS), which is facilitated by the enzyme serine palmitoyltransferase (SPT).[34] 3-ketoreductase reduces 3-KDS to form dihydrosphingosine, an intermediate possessing a sphingosine base. In the next step, a fatty acyl group is added to dihydrosphingosine by dihydroceramide synthase to generate dihydroceramide. Finally, the enzyme dihydroceramide desaturase 1 (DES1) inserts a double bond into the sphingosine base of dihydroceramide to form ceramide. Ceramide can then be further transformed into ceramide-1 phosphate, acyl ceramide, sphingomyelin, glycosphingolipids, or sphingosine.[21,35] Like sphingosine, each ceramide is generated from combinations of sphingosine bases and fatty acids and performs unique functions.[37,38] Ceramide production is tightly controlled by ORMDL family proteins, which sense ceramide levels and inhibit SPT. Since sphingosine can only be formed from ceramide, ORMDL proteins regulate sphingolipid levels.[35]

To generate S1P, ceramide is transported by ceramide transfer protein to the Golgi apparatus, where polar head groups are added.[34] Ceramide possesses a sphingolipid core, which, when N-deacetylated by ceramidase, is broken down to sphingosine and a free fatty acid (FFA).[39] Sphingosine is then phosphorylated by sphingosine kinases (SphKs) to form S1P. Two isoforms of SphK have been identified and characterized. SphK1 is predominantly localized in the cytosol. SphK2 is mainly in the nucleus and mitochondria.[39,40] Sphingosine can be converted back to ceramide via CerS, which is typically localized near the ER but is also found in the nuclear membrane and mitochondria.[21] Tissue concentrations of S1P remain low since most synthesized S1P is secreted or dephosphorylated by S1P phosphatase or catabolized by S1P lyase[21] (Figure 3). The intracellular S1P is transported to the extracellular space by a specific transporter in the cell membrane, either spinster homolog 2 or ATP binding cassette transporters.

Sphingolipids are not only important structural components of lipid membrane but also important signaling molecules. S1P is the most well-characterized signaling molecule. It can directly act as an

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**Figure 3:** Synthetic pathways of sphingolipids. The ceramide is the core molecule of sphingolipids. It can be formed from an amino acid, serine, and palmitoyl-CoA, by de-novo synthesis or from sphingomyelin via sphingomyelinase or from sphingosine by ceramidase. Ceramide can be converted to C1P by ceramide kinase. Sphingosine can be phosphorylated by sphingosine kinase to form S1P, which can be dephosphorylated by SGPP or further metabolized by SGPL to hexadecenal + ethanolamine-1 phosphate. C1P: Ceramide-1 phosphate; SGPP: S1P phosphatase; SGPL: Sphingosine phosphate lyase; S1P: Sphingosine-1 phosphate.
intracellular signaling molecule or extracellularly via five GPCRs, S1PR1-5, which are differentially expressed in multiple tissues and cells and can activate various signaling pathways via coupling with different G-proteins (Figure 4).

**Bile Acids and Sphingolipids in NAFLD**

The liver is physiologically equipped to optimize energy usage necessary for survival under primitive conditions. As such, the liver restricts circulating lipid levels by serving as a reservoir for short-term lipid storage. In metabolic associated NAFLD, adipocyte hypertrophy and insulin resistance induce lipolysis and increase serum FFA, promoting lipid uptake into hepatocytes and other organ tissue, driving steatosis. Although not completely understood, the onset of inflammation that drives the progression from NAFL to NASH is attributed to a mosaic of factors, including gut dysbiosis, genetics, and diet. Pro-inflammatory conditions accelerate the progression from NAFL to NASH, characterized by hepatocyte injury (ballooning) and hepatic inflammation in the presence of steatosis. As NAFLD progresses, the liver expands in size due to the excessive uptake of fats. With repeated insult due to chronic inflammation, resident cell populations may be gradually replaced with non-functional, fibrotic tissue. In NAFLD, hepatic stellate cells (HSCs) differentiate to a myofibroblast-like phenotype that enables the excessive production of extracellular matrix (ECM) components. ECM is a driver of progressive fibrosis. Reactive oxygen species (ROS), cytokines, and chemokines, especially transforming growth factor-β (TGFβ) and tissue inhibitor of matrix metalloproteinase 1 (TIMP1), inhibit matrix metalloproteinases that degrade ECM, promoting fibrogenesis. In the recent decade, extensive studies from animal models and human patients have found that NAFLD disease progression is closely associated with bile acid dysregulation and sphingolipid homeostasis.

**Mechanisms linking bile acid metabolism to the development of NAFLD**

Consistently, clinical studies have demonstrated that primary and secondary CBAs are tightly associated with NAFLD progression and NASH severity.
Mechanisms linking sphingolipid metabolism to the development of NAFLD

Sphingolipids are important signaling molecules and have been extensively studied in various human diseases, including metabolic diseases and NAFLD.\(^\text{61,62}\) Ceramide and SIP are the two most well-characterized sphingolipids. Lipid profiling studies have shown that increased ceramide is closely correlated with the pathogenesis of metabolic diseases, such as obesity and insulin resistance in animal models.\(^\text{20}\) The metabolomics and lipidomics analysis of human patient samples also indicate the association of ceramide levels in the serum and tissue with obesity, NAFL, and NASH.\(^\text{20}\) The key enzymes involved in sphingolipid metabolism have been studied as therapeutic targets for metabolic diseases. Inhibition of SPT with Myriocin, a potent SPT inhibitor, or deletion of Sptlc1 or Sptlc2, has been studied in animal models for modulating insulin sensitivity, hepatic steatosis, and adipocyte function.\(^\text{20}\) In addition, the DES1 inhibitor, fenretinide, has been reported to resolve insulin resistance and hepatic steatosis in obese mice.\(^\text{63}\) However, another study reported that fenretinide accelerated atherosclerosis in apoE-deficient mice.\(^\text{64}\) More studies are needed to delineate the potential mechanisms underlying the differential effects of the DES1 inhibitor.

It has been well characterized that the genetic risk variant of patatin-like phospholipase domain-containing protein 3 (PNPLA3)\(^\text{I148M}\) has a significant impact on NAFLD disease severity in both pediatric and adult patients. It also has been reported that NASH patients carrying the PNPLA3 mutant are predisposed to HCC development.\(^\text{65}\) A recent study using the well-characterized western diet and sugar water (WDSW)-induced NASH mouse model showed that hepatocyte-specific overexpression of human PNPLA3\(^\text{I148M}\) accelerated NASH progression with increased steatosis, inflammation, fibrosis, and HCC.\(^\text{66}\) Interestingly, the transcriptomic and metabolomic analysis showed that the ceramide level was markedly upregulated in PNPLA3\(^\text{I148M}\) mice upon WDSW feeding, which was accompanied by significant changes in sphingolipid metabolism and signaling pathways.\(^\text{66}\) The key genes involved in ceramide synthesis, sphingomyelin and salvage pathways were markedly increased, such as Sptlc1/2, Kdsr, Smpd1/2, and Cers.\(^\text{66}\) This study further indicated the potential role of sphingolipids in NAFLD pathogenesis.

Inflammation and fibrosis are two major driving forces of NAFL to NASH-HCC progression. SIP is the most well-characterized sphingolipids and has been extensively studied for its role in inflammation.\(^\text{67}\) SIP can directly activate intracellular signaling pathways or via five recent study reported that elevated hepatic taurochenodeoxycholic acid (TCDCA) and taurocholic acid (TCA) in NAFLD patients was associated with symptom onset, disease progression, or death.\(^\text{53-55}\) A similar study demonstrated that serum CBA elevations are not observed in NAFL and occur in both non-obese and obese patient cohorts.\(^\text{56}\) Although NAFLD is not correlated to cholestasis, increases in primary CBAs, especially TCA, and a decrease in secondary bile acids, have been observed.\(^\text{48}\) In a similar study where the blood chemistries of 102 NAFLD and 50 healthy patients were monitored, NAFLD patients exhibited highly significant elevations in plasma total, primary, and secondary bile acids. Changes in bile acid composition were significantly correlated with steatosis. Increased primary CBAs, TCA, taurodeoxycholic acid, TCDCA, glycocholic acid (GCA), and glycodeoxycholic acid, were associated with hepatic inflammation. Interestingly, GCA and glycine chenodeoxycholic acid (GCDC) correlated with fibrosis severity and point mutations in the tribbles homolog 1 gene, which functions as a negative regulator of retinoic acid receptors.\(^\text{49}\) These patient data strongly suggest that dysregulation of bile acid homeostasis represents a key mechanism underlying inflammation and fibrosis in NAFLD. Importantly, patient genetic profiling is an essential consideration for data stratification in future studies and may provide opportunities for novel pharmacogenomic therapies.

Our previous studies showed that bile acids play a crucial role in hepatic lipid metabolism through activation of S1PR2. In the primary CBAs, such as TCA, GCA, GCDC, and TCDCA, can activate extracellular-signal-regulated kinase (ERK)1/2 and protein kinase B (AKT) pathways via S1PR2.\(^\text{53-55}\) Both S1PR2 and SphK2 knockout (S1PR2\(^{−/−}\) and SphK2\(^{−/−}\)) mice were prone to develop fatty liver upon high-fat diet feeding.\(^\text{56}\) By using the chronic bile fistula rat model and S1PR2\(^{−/−}\) and SphK2\(^{−/−}\) mouse models, we further identified that TCA-induced activation of S1PR2 induced expression and activation of SphK2, which results in the generation of nuclear S1P.\(^\text{56}\) Nuclear S1P has been identified as a potent inhibitor of histone deacetylases (HDACs).\(^\text{57}\) HDACs have been recently recognized as important pathogenic players in NAFLD.\(^\text{34}\) However, due to the broad regulator effect on gene transcription, targeting HDACs alone may not be feasible as the treatment for NAFLD.\(^\text{58}\)

Since the identification of FXR as the first bile acid nuclear receptor and TGR5 as the first bile acid-activated GPCR, extensive studies have been done to examine the therapeutic potential for NAFLD by modulating FXR and TGR5 activity. The role of FXR and TGR5 in NAFLD has been recently reviewed.\(^\text{52}\) Several bile acid-like drugs have been evaluated in clinical trials. In 2020, obeticholic acid, an FXR agonist, was evaluated for the treatment of NASH associated fibrosis. Due to incomplete review, uncertain long-term outcomes, and the possible risk of liver failure, it did not progress past phase III trials despite fibrosis improvement.\(^\text{59}\)

A recent study reported that Sevelamer, a bile acid sequestrant, reversed liver injury and prevented NASH development in a high-fat and high-sucrose diet-induced mouse NAFLD model.\(^\text{60}\) Sevelamer is a non-absorbable polymer that not only binds bile acids in the intestine to form non-absorbable complexes and reduces bile acid uptake by intestinal epithelial cells but also modulates the gut microbiome and bile acid metabolism.\(^\text{61}\) The role of bile acids and gut microbiome in NAFLD has also been reviewed recently.\(^\text{12}\)
GPCRs to regulate various physiological and pathological processes. It has been shown that S1P-mediated activation of S1PR1/S1PR3 activates HSCs and promotes HSC migration and differentiation into myofibroblasts, enabling excessive ECM protein deposition. Activation of hepatic S1PR1 led to nuclear factor kappa B activation and increased chemokine and cytokine production in a diet-induced NASH mouse model. A previous study identified the activation of the GP130-STAT3 signaling pathway in human NASH patients. In the recent study, multiple inflammatory pathways were activated, including activation of the Janus kinase-STAT3 in the PNPLA3/NASH mouse model. Additionally, *in vitro* studies further showed that the culture of human HSCs, LX2 cells, with the conditioned media from HepG2 hepatocytes, which express PNPLA3, significantly induced TGF-β1 and procollagen I and III expression. These studies suggest that sphingolipid-mediated signaling in hepatocytes activates the fibrogenic response in NALFD.

Sphingolipid interactions are further nuanced by signal localization and cell type. The isoforms Sphk1 and Sphk2 have divergent roles in NALFD pathology; this is likely due to the subcellular localization of each enzyme. Sphk1 is predominantly localized in the cytosol. Sphk2 is mainly in the nucleus and mitochondria. S1P generated by Sphk1 is involved in pro-inflammatory signaling through S1PR1-3. After synthesis, S1P is exported from the cell and interacts with S1PR1-5. Each GPCR mediates pathways linked to Ras family small GTPases, Rac and Rho, and protein kinases, including p38/MAPK, PI3K/AKT, JNK, and phospholipase Ca²⁺/inositol triphosphate/diacylglycerol. Sphk1 is involved in pro-inflammatory pathways clarifying how adipocyte Sphk1 deletion increases hepatic lipid accumulation are not fully understood, these contributions highlight the interconnectivity between adipocytes Sphk1 and liver function. These data are especially important to consider in future metabolic and NASH studies.

**Summary and Future Perspectives**

Despite NALFD being one of the fastest growing diseases, continued research is needed to elucidate the biological mechanisms underlying NALFD to develop novel therapeutic interventions. Multiple recent reviews have summarized the potential therapeutic and diagnostic value in targeting components of bile acid and sphingolipid metabolism. Therapeutics which target different components of bile acid metabolism can have unanticipated effects on glucose homeostasis or lipid metabolism, but adverse side effects are mostly isolated to gastrointestinal dysregulation. However, bile acid pathways appear to be a sensible pharmacologic target. Bile acids tightly correlate with NALFD severity and BA-driven mechanisms regulate hepatic lipid uptake, inflammation, and fibrosis.

Future studies may benefit from considering subtle structural differences in bile acids and sphingolipids, as well as the localization of these signaling molecules. In addition, the role of sphingolipids in the cross-talk of gut and liver has not been well studied, especially the impact of sphingolipids on gut microbiomes and intestinal barrier function. Furthermore, incorporating personalized analysis of specific genetic risk factors and underlying enterohpatic circulation abnormalities may improve diagnosis and treatment for NALFD.

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**Conflicts of interest**

None.

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