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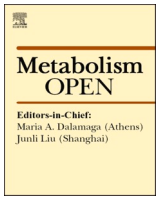

# Drug targets regulate systemic metabolism and provide new horizons to treat nonalcoholic steatohepatitis

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ABSTRACT

Nonalcoholic steatohepatitis (NASH), is the advanced stage of nonalcoholic fatty liver disease (NAFLD) with rapidly rising global prevalence. It is featured with severe hepatocyte apoptosis, inflammation and hepatic lipogenesis. The drugs directly targeting the processes of steatosis, inflammation and fibrosis are currently under clinical investigation. Nevertheless, the long-term ineffectiveness and remarkable adverse effects are well documented, and new concepts are required to tackle with the root causes of NASH progression. We critically assess the recently validated drug targets that regulate the systemic metabolism to ameliorate NASH. Thermogenesis promoted by mitochondrial uncouplers restores systemic energy expenditure. Furthermore, regulation of mitochondrial proteases and proteins that are pivotal for intracellular metabolic homeostasis normalize mitochondrial function. Secreted proteins also improve systemic metabolism, and NASH is ameliorated by agonizing receptors of secreted proteins with small molecules. We analyze the drug design, the advantages and shortcomings of these novel drug candidates. Meanwhile, the structural modification of current NASH therapeutics significantly increased their selectivity, efficacy and safety. Furthermore, the arising CRISPR-Cas9 screen strategy on liver organoids has enabled the identification of new genes that mediate lipid metabolism, which may serve as promising drug targets. In summary, this article discusses the in-depth novel mechanisms and the multidisciplinary approaches, and they provide new horizons to treat NASH.

## 1. Introduction

### 1.1. The high prevalence of global NASH and limited treatment options

Nonalcoholic fatty liver disease (NAFLD) is a common type of metabolic disorder, which is characterized by excessive lipid accumulation in liver due to abnormal genetic manifestation, unhealthy lifestyle (intake of high fat and caloric food) and medication [1]. The global prevalence of NAFLD is estimated between 25 and 38 % [2]. Among the different stages of NAFLD, nonalcoholic steatohepatitis (NASH) is the advanced stage of NAFLD, which is featured with cellular injury (ballooned hepatocytes), significant hepatic steatosis and lobular inflammation. The global prevalence of NASH is estimated between 2 and 6%. NASH can progressively develop into liver fibrosis and subsequently cirrhosis, and these transformations are irreversible with incidence between 0.5 and 2.6 % per year globally. They eventually transform to NAFLD-related hepatocellular carcinoma (HCC), while HCC is notoriously lacking of effective treatment and has poor prognosis [3].

The effective therapeutic options for NASH treatment are limited and are not curative, which include surgery and change in lifestyle. Among the strategies in surgery, liver transplantation is the major therapeutic option for the advanced stage of NASH. Nevertheless, the number of adults with NASH awaiting liver transplantation has almost tripled since 2004 in the USA, and thus the therapy is limited by the shortage of donors [4]. Moreover, recipients that receive liver transplantation also experience metabolic complications and recurrence of the disease, thereby requiring lifestyle modification, management of immunosuppressant drugs and treatment of metabolic complications [5]. In contrast, bariatric surgery (gastric bypass and other weight-loss surgeries) of severe obese subjects induced long-term improvement of NASH and fibrosis [6], in which fibrosis decreased and even

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**Abbreviations**

| | |
|---|---|
| AAC | ADP/ATP carrier |
| AAV | Adeno associated virus |
| AdipoR | Adiponectin receptor |
| ALT | Alanine aminotransferase |
| AMPK | AMP-activated protein kinase |
| BTK | Bruton's tyrosine kinase |
| ClpXP | Caseinolytic protease |
| CNS | Central nervous system |
| Cryo-EM | Cryo-electron microscopy |
| DDX5 | DEAD-box protein 5 |
| DGAT2 | Diacylglycerol acyltransferase 2 |
| DNP | 2,4-dinitrophenol |
| ER | Endoplasmic reticulum |
| FGF21 | Fibroblast growth factor 21 |
| FXR | Farnesoid X receptor |
| GLP-1 | Glucagon-like peptides 1 |
| HCC | Hepatocellular carcinoma |
| HFD | High-fat diet |
| IRE1α | Inositol-requiring transmembrane kinase endoribonuclease-1α |
| MCD | Methionine/choline–deficient diet |
| mTOR | Mechanistic target of rapamycin complex |
| NAFLD | Nonalcoholic fatty liver disease |
| NAS | NAFLD activity score |
| NASH | Nonalcoholic steatohepatitis |
| OCA | Obeticholic acid |
| PPAR | Peroxisome proliferator-activated receptor |
| RBP4 | Retinol binding protein 4 |
| ROS | Reactive oxygen species |
| sEH | Soluble epoxide hydrolase |
| SNP | Single nucleotide polymorphism |
| TG | Triglyceride |
| TGR5 | Takeda G-protein-coupled receptor 5 |
| THRβ | Thyroid hormone receptor β |
| UCP1 | Uncoupling protein-1 |
| Xbp1 | X-box binding protein-1 |

disappeared. In these patients, the median value of NAFLD activity score (NAS) decreased from 5 to 1, and the value of most patients improved by at least 2 points of NAS. It indicates the restoration of systemic metabolic homeostasis and energy expenditure are important for NASH resolution.

### *1.2. The current development of small molecule NASH therapeutics*

In parallel to the progress in surgery and lifestyle change, the past two decades have witnessed the rapid growth in the elucidation of the detailed mechanism of NASH pathogenesis by identification and validation of the genes and their interacting networks that promote NASH. As recently reviewed in multiple studies, the small molecule modulators to treat NASH are thus continuously developed to directly inhibit the function of targets that regulate apoptosis, hepatic steatosis, inflammation and fibrosis [7–11]. Here we summarize the drugs tested in phase II and III clinical trials and their mechanism of action in Table 1. Clearly, the small molecules targeting these pathways have already revealed promising anti-NASH potencies in preclinical and clinical studies. Nevertheless, currently there are no FDA-approved pharmacological treatments for NASH.

### *1.3. The problem of currently failed NASH therapeutics*

The significant challenges in development of NASH therapeutics can be attributed to multiple causes. The previously unappreciated pathogenic pathways have been revealed to contribute to the compromised efficacy of NASH therapeutics. For instance, hepatic FXR is a type II nuclear receptor and ligand-activated transcription factor. It regulates bile acid metabolism, along with a wide range of physiological functions [30]. Activation of FXR downregulates lipid synthesis and alleviates inflammation partially through suppression of NF-κB binding to promoters of FXR-regulated genes [31,32]. The most prominent FXR agonist, OCA, is currently tested in phase III clinical trial to treat NASH [33]. Yet, the mechanism-based adverse effects of steroidal FXR agonists, e.g. pruritus and elevated low-density lipoprotein cholesterol are the major concerns of their application in NASH treatment [34]. Biochemical study indicates that activated hepatic stellate cells enhance SUMOylation of FXR (a kind of post-translational modification), and thus limit the activation of FXR to OCA and other FXR agonists [35]. Moreover, treating fibrosis is not effective for most patients by current NASH therapeutics [24,36]. For instance, simtuzumab, the monoclonal antibody against lysyl oxidase-like 2 (LOXL2), and selonsertib, the apoptosis signal-regulating kinase 1 (ASK1) inhibitor were unable to reverse bridging fibrosis or compensated cirrhosis associated with NASH [15,24]. In the phase III clinical study, selonsertib effectively downregulated the level of phosphorylated p-38 in liver of patients, which is the downstream effector of ASK1 signaling pathway. Nevertheless, fibrosis improvement was only achieved in a small portion of patients with NASH (around 10%) [24]. Furthermore, no significant reduction in hepatic collagen content, a hallmark of liver fibrosis, was observed between patients receiving simtuzumab 75 mg and those receiving placebo [36]. In summary, the current understanding of the complexity of NASH pathogenesis is only the tip of iceberg.

Secondly, genetic variations in patients, as represented by single nucleotide polymorphisms (SNP) in pathogenic genes, determine the progression of NASH and response to therapeutics. Thus, the efficacy and objective response in patients with NASH are heterogeneous [37]. The heterogeneity of response to NASH therapeutics in patients poses great challenges, and these challenges should be addressed in preclinical studies. Nevertheless, current murine models are not perfect in recapitulating the natural history and complex genetics of NASH, as they exhibit limited genetic diversity [38]. It is thus difficult to represent a highly diversified genetic variability present in human populations. Therefore, the conclusion drawn from the murine models may not accurate reflect the real processes of NASH pathogenesis in patients.

Thirdly, the failure of small molecules can be attributed to obvious adverse effects that impede the escalation of their doses to cure NASH. For instance, as FXR can regulate the expression of a myriad of genes, the non-selective activation of FXR by canonical agonists (e.g. OCA) generates unwanted side effects, such as pruritus and dyslipidemia in patients with NASH [39]. Moreover, treatment of FXR agonist cilofexor is positively correlated with elevated IL-31 level and pruritus adverse events in patients with NASH [40]. These adverse effects collectively urge the in-depth understanding of the significant challenges in NASH treatment and identification of new therapeutic targets.

### *1.4. The emerging new horizons to treat NASH by targeting root causes*

As we can see in Table 1, there are multiple solutions to address the challenges in pharmacological treatment of NASH. The first is to improve the efficacy of current therapeutics by designing drugs that target multiple proteins. The molecular and genetic traits of the hubs in NASH determine the NAFLD-NASH transition, fibrosis and NASH-HCC transition [41–43]. Thus, drugs targeting the multiple molecular hubs of the metabolic networks simultaneously may be superior to the current monotherapies (such as cenicriviroc in Table 1) [44]. Secondly, new

**Table 1**
Summary of the drugs tested at phase II and III trials, their targets and mechanism of action to treat NASH (Since 2020).

| Drug | Target and mechanism | Efficacy | Stage | Reference |
|---|---|---|---|---|
| Aldafermin | An engineered analog of fibroblast growth factor 19, and inhibits bile acid synthesis and regulates metabolic homeostasis | No significant dose response on fibrosis improvement was observed. | IIb | [12] |
| Aldafermin | An engineered analog of fibroblast growth factor 19, inhibits bile acid synthesis and regulates metabolic homeostasis | It significant reduces absolute liver triglyceride and cholesterol content. It reduces ALT level. 38 % of patients in the aldafermin group achieved fibrosis improvement with no worsening of NASH. | II | [13] |
| Apararenone | A mineralocorticoid receptor agonist | It reduces ALT level and fibrosis markers, such as type IV collagen 7S and procollagen-3 N-terminal peptide. | II | [14] |
| Aramchol | A partial inhibitor of hepatic stearoyl-CoA desaturase (SCD1) to inhibit lipogenesis | It decreases hepatic triglycerides. The fibrosis is improved by $\geq$ 1 stage without worsening NASH in 29.5 % patients. | IIb | [15] |
| ARO-HSD | An oligonucleotide to reduce the mRNA of HSD17β13 and blocks lipogenesis | It reduces hepatic HSD17β13 mRNA and protein expression, which are accompanied by reduction in alanine aminotransferase (ALT). Nevertheless, ARO-HSD treatment doesn't significantly reduce liver fat fraction in patients. | I/II | [16] |
| Cenicriviroc | A dual antagonist for C–C chemokine receptors type 2 and 5 | It ameliorates liver fibrosis by achieving fibrosis response at year 1 maintained benefit at year 2. It reduces N-terminal type 3 collagen propeptide in patients. | IIb | [17] |
| Cilofexor | A farnesoid X receptor (FXR) agonist | It significant reduces hepatic steatosis, liver biochemistry, and serum bile acids in patients with NASH. Nevertheless, it doesn't reduce markers of fibrosis. | II | [18] |
| EDP-305 | A farnesoid X receptor (FXR) agonist | It reduces ALT level and liver triglyceride content. It also reduces circulating levels of the bile acid precursor C4. | II | [19] |
| Efruxifermin | A Fc-FGF21 fusion protein and normalizes systemic glucose and lipid metabolism | It improves the markers of liver injury, and glucose and lipid metabolism by 16-week treatment. It significantly reduces markers of fibrosis, such as serum Pro-C3. | IIa | [20] |
| Lanifibranor | A pan-PPAR agonist to resolve NASH | Patients had decrease at least 2 points in the SAF-A score without worsening of fibrosis. | IIb | [21] |
| Rencofilstat | A cyclophilin inhibitor to counteract the hepatotoxic effects of steatosis | It reduces ALT level and fibrosis by suppression of collagen deposition. Key collagen genes are reduced. | IIa | [22] |
| Resmetirom | A thyroid hormone receptor agonist | It reduces LDL-C, aopB, triglycerides, etc | III | [23] |
| Selonsertib | An ASK1 inhibitor to block liver injury, inflammation and fibrosis | It is ineffective to block fibrosis in patients with bridging fibrosis or compensated cirrhosis due to NASH. | III | [24] |
| Semaglutide | A GLP-1 receptor agonist | It resolves NASH in significant portion of patients. | II | [25] |
| Tropifexor | A farnesoid X receptor (FXR) agonist to block steatosis, inflammation and fibrosis | It reduces ALT level, aspartate aminotransferase (AST) level and hepatic fat fraction (HFF). | IIa/b | [26] |
| Tropifexor plus cenicriviroc | Activate Farnesoid X receptor (FXR) and C–C chemokine receptors type 2 and 5 simultaneously | No substantial incremental efficacy compared with cenicriviroc monotherapy was observed. | IIb | [27] |
| TVB-2640 | A fatty acid synthase inhibitor to reduce excess liver fat and it directly inhibits inflammatory and fibrogenic pathways | It reduces liver triglyceride content at week 12. It also reduces ALT level, aspartate aminotransferase, and γ-glutamyl transpeptidase. | IIa | [28] |
| Volixibat | An inhibitor of the apical sodium-dependent bile acid transporter to block bile acid reuptake and the hepatic bile acid production | Volixibat doesn't reduce liver fat fraction, nor serum ALT levels. It increases bile acid precursor C4 level and decreases total and low-density lipoprotein cholesterol. | II | [29] |

antifibrotic approaches have been proposed [7]. Integration of anti-inflammatory and anti-fibrotic strategies are promising options to reverse NASH progression [10]. Moreover, the combination of therapies and exercise alternatively address the challenges [8,9]. Nevertheless, the functional redundancy in the signaling pathways of multiple interventions may compromise the efficacy (see Tropifexor and cenicriviroc in Table 1). Thirdly, the efficacy and safety of current NASH therapeutics can be further improved by means of medicinal chemistry.

A *Nat Rev Gastroenterol Hepatol* article had drawn the fundamental conclusion as "treatment of NASH should be anchored in its ***root cause***" [45]. The authors defined the root cause of NASH as the "lipotoxic injury to the liver or has pleiotropic effects at different points in the disease cascade". The new NASH therapeutics thus should target the root cause in multiple aspects. As a great portion of patients with NASH have metabolic comorbidities as well, the root cause drives the dysregulated energy expenditure of whole body. Identification of new drug targets that address the root cause in NASH and elucidation of their action mechanisms have gradually been appreciated. They may change the landscape of NASH therapeutics. What are the new horizons in treatment of NASH?

The past three years have witnessed the new trend in drug discovery for NASH therapeutics. New targets may not directly intervene with inflammatory, fibrotic or lipogenic pathways. Instead, their modulation by drugs normalizes the systemic metabolic homeostasis, and eventually inhibits most of the prominent pathogenic pathways. In this new paradigm, the therapeutics act on their binding proteins expressed in tissues or organs other than liver to improve glucose and lipid metabolism. It has exhibited remarkable improvement in NASH symptoms, as well as the co-existed metabolic complications in human subjects. The pilot studies are the recent trial of GLP-1 receptor agonists, such as semaglutide. Semaglutide mimicked the function of endogenous incretin, GLP-1 and it manifested superior anti-obesity and anti-diabetic activities, and its administration induced resolution of NASH in 59% of patients (no more than mild residual inflammatory cells [score of 0 or 1], no hepatocyte ballooning [score of 0] and no worsening of liver fibrosis) [25]. Moreover, the Phase IIb trial with combination of a GLP-1 receptor agonist and Efruxifermin (Fc-FGF21 fusion protein) reduces liver fat in patients with NASH (*Nature Medicine* News, Aug 29, 2023).

The application of GLP-1 receptor agonists in treatment of NASH revolutionizes the development of NASH therapeutics. In addition to semaglutide, the dual agonist for GLP-1 and GIP receptors, tirzepatide (developed by Eli Lilly) exhibited remarkable anti-NASH activity and significantly decreased NASH-related biomarkers in patients with diabetes [10,46]. In preclinical studies, small molecule GLP-1 receptor agonists, such as cinchonine also improved liver function in mice with NASH [47]. Thus, the drugs that recapitulate the function of "beneficial" secreted proteins *in vivo* may be new horizon in NASH treatment.

In addition to systemic improvement, normalization of mitochondrial function in liver cells effectively eliminates the excessive hepatic lipids and restores the intracellular energy homeostasis. It reverses

NASH progression. Activation of mitochondrial pyruvate carrier (MPC) by small molecules effectively enhances the catabolism of branched chain amino acids and improves metabolic complications in patients and rodents [48]. Moreover, Deuterium-stabilized (R)-pioglitazone (PXL065) activates MPC and PXL065-treated patients displayed histological improvement and restoration of systemic metabolic homeostasis, which is independent of its canonical PPARγ-induced effects [49,50]. These proof-of-concept studies may provide new insights into addressing the "root cause" of NASH progression.

In the article, we will in-depth discuss the recent findings in treatment of NASH through targeting the root causes of NASH. These mechanisms of action are beyond the well-studied direct intervention of lipogenesis, inflammation and fibrosis. Furthermore, the multidisciplinary advances that facilitate the development of small molecule modulators, their pros and cons will be critically analyzed. The framework of this article is illustrated in Fig. 1.

## 2. The new insights in therapeutics that target well-studied NASH targets

Structural modification of drugs is guided by advances in structural biology and medicinal chemistry. It has generated potent small molecule modulators with higher selectivity, stability and safety. The improvement of current NASH drugs may thus address the challenges in ineffectiveness and adverse effects.

### *2.1. Novel farnesoid X receptor (FXR) agonists stabilize the unique conformation of FXR*

The recent findings have indicated that different FXR conformation will result in recruitment of distinct co-activators and co-repressors to stimulate the expression of downstream genes [51]. Thus, novel agonists with biased agonism partially activate some of the FXR downstream effectors through inducing unique FXR conformation, and thus they minimize the adverse effects. For instance, non-bile acid agonist MET409 has biased agonism. It improves the symptoms of NASH, as 12-week administration of MET409 lowers liver fat content by 55% and improves liver function. Moreover, the adverse effects in patients with NASH is greatly diminished compared with those of traditional therapies (Fig. 2, Upper left) [52].

### *2.2. TGR5 agonists specifically locate in intestine to enhance efficacy*

Takeda G-protein-coupled receptor (TGR5) is a member of the rhodopsin-like subfamily of GPCRs and it is expressed on cell surface. It regulates a myriad of physiological processes via binding to the endogenous bile acids. Activation of TGR5 expressed on the cell membrane of liver macrophages attenuates inflammation via NLRP3 signaling pathway [53]. In addition, activation of intestinal TGR5 in enteroendocrine cells by bile acids stimulates the secretion of glucagon-like peptides 1 and 2 (GLP-1, 2) to regulate the homeostasis of glucose metabolism [54,55]. The small molecules with dual activation of TGR5 and FXR have shown excellent efficacy in resolution of NASH in mice model induced by HFD (High-fat diet) and $CCl_4$. Notably, liver NAS score, fibrosis were much improved and triglyceride (TG) levels were downregulated [56]. Nevertheless, administration of the previously developed TGR5 agonists caused their distribution throughout the bloodstream. It leads to systemic activation of TGR5 and elicits adverse effect, e.g., excessive gallbladder filling and blockade of gallbladder emptying [57].

Advances in medicinal chemistry have provided new TGR5 agonists with special pharmacophores. Pharmacophores such as thiazolidine, D-glucamine and quinoxaline moieties are introduced into the TGR5 agonists, and the modified agonists are retained in intestine. Thus, their specificity and efficacy are improved, leading to more robust and sustained secretion of GLP-1, when compared with those of traditional TGR5 agonists. Importantly, they avoid the adverse effects induced by systemic distribution (Fig. 2, Lower left) [58].

### *2.3. Optimized agonists for PPAR lower the toxicity and increase the potencies*

Activation of peroxisome proliferator-activated receptors (PPARs) effectively resolves NASH in murine model [59]. PPARδ agonist induces hepatic autophagy, and thereby enhancing fatty acid oxidation and reducing lipid accumulation through AMPK/mTOR pathway [60]. Nevertheless, potent dual PPARα/δ agonist, GFT505 (Elafibranor) was unable to block liver fibrosis and thus failed in phase III clinical trial. Its hepatotoxicity restrained the dose escalation to resolve NASH. The structural optimization generates derivative **3d**, which substitutes the previous methyl sulfide of GFT505 with methylamine group, and free carboxylic acid with *tert*-butyl ester (see Fig. 2, Upper right, for the optimization) [61]. These structural optimizations effectively lowered the binding energy of **3d** to PPARα and δ, and stabilized the **3d**-PPAR complex. Consistently, **3d** effectively lowered the hepatotoxicity in murine NASH model, along with superior liver index compared with that of GFT505. Moreover, **3d** resolved hyperlipidemia, liver fat degeneration and liver inflammation with higher efficacy compared with GFT505 at the same doses in methionine-choline deficiency (MCD)-induced murine NASH model [61].

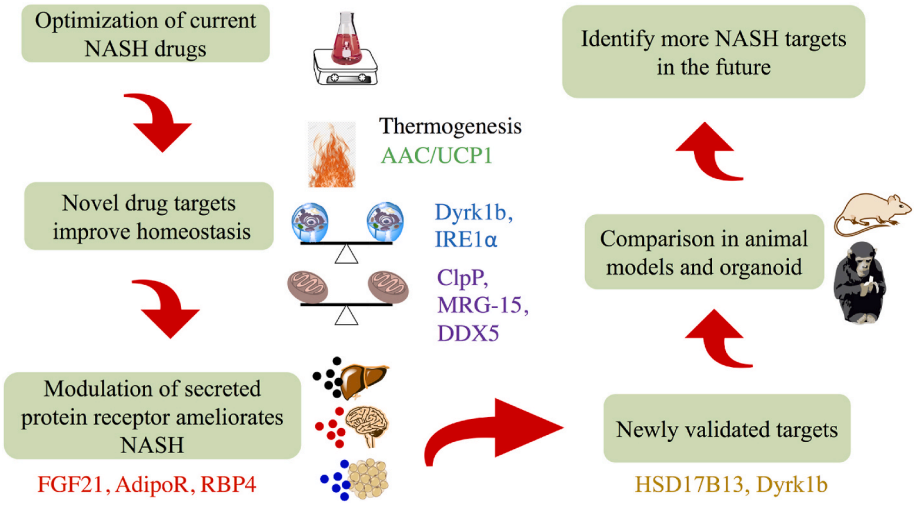


**Fig. 1.** Framework of the whole article and schematic illustration of the emerging drug targets and strategies that will be focused on in the article.

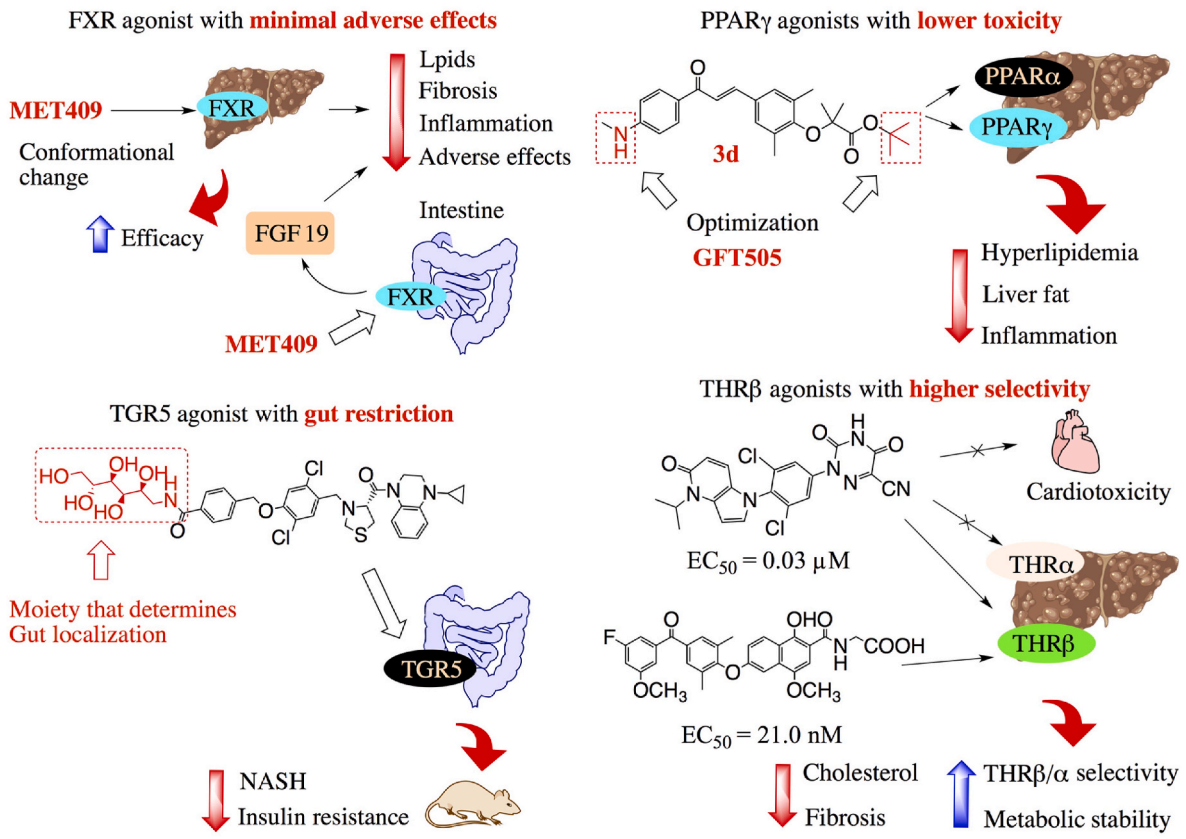


**Fig. 2.** Optimization of current NASH drugs increases potency and selectivity. Upper left: Optimized FXR agonists MET409 selectively stabilize conformation of FXR and activate FXR signaling transduction; Below left: TGR5 agonist with intestine-directing group (labeled with red) specifically targets TGR5 in intestine and alleviates metabolic complications; Upper right: Optimization of PPARα/δ agonist GFT505 lowers toxicity and metabolic complications (The optimized moieties are labeled with red); Below right: Optimization of THRβ receptor agonists increases the selectivity, lowers the cardiotoxicity and enhances the potencies. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

### *2.4. Thyroid hormone receptor β (THRβ) agonists: increasing the selectivity*

Thyroid function is closely associated with NAFLD progression and activation of thyroid hormone receptors alleviates NASH and hepatic fibrosis in murine model [62,63]. THRβ agonists are the third class of nuclear factor agonists that reduce cholesterol and triglyceride by suppression of the genes in promoting their biosynthesis [64]. Notably, in a phase II clinical trial (Table 1), Resmetirom (MGL-3196) significantly reduces the hepatic lipid accumulation in human subjects with NASH [65]. Recent phase III trial study has indicated its good tolerance and reduction of LDL-C, aopB, and triglycerides [23]. In contrast to Resmetirom, another liver-selective thyroid hormone receptor agonist, eprotirome was terminated prematurely in phase III clinical trials due to the adverse effects: cartilage damage (Table 1) [66]. Thus, the side effects of THR agonists should be cautiously determined.

The recent agonists exhibit advantages in THRα/β subtype selectivity. The only amino acid difference in ligand-binding pockets between THRα and THRβ accounts for the suboptimal subtype selectivity of previous THRβ agonists [67], thereby increasing the adverse effects in heart and bone by activation of THRα [68]. The optimized compounds are designed based upon the crystal structure between THRβ and the analog of MGL-3196. The most potent compound undergoes two stages of optimization: In the first stage, the cyclization of MGL-3196 forms indole ring and exhibits 30-fold higher efficacy against THRβ, compared with MGL-3196 ($EC_{50}$ = 0.03 μM vs 0.99 μM of MGL-3196). In the second stage, the substitution on indole ring with N atom generates pyrrolo[3,2-b]pyridin-5-one skeleton, which yields much higher THRβ/α selectivity (11.2-fold vs 4.2-fold) and liver-to-serum ratio (93:1 vs 6:1) [68]. The single oral dose of new compound in murine model effectively lowers total cholesterol and LDL-cholesterol levels in serum. Meanwhile, liver steatosis, inflammation, especially fibrosis is substantially ameliorated in HFD-combined $CCl_4$-induced mice model of NASH (Fig. 2, Lower right). The high THRβ/α selectivity of the agonists and their selective enrichment in liver ensure no cardiotoxicity caused by nonspecific activation of THRα.

The histidine and phenylalanine residues in THRβ form hydrogen bonds with its agonists, and the binding leads to the low THRβ/α selectivity (so-called His-Phe switch). Recently, an alternative novel strategy breaks the "His-Phe switch". A series of (4-hydroxy-1-alkoxyisoquino- line-3-carbonyl) glycine derivatives generate the potent THRβ agonist with $EC_{50}$ at 21.0 nM [67]. It shows excellent metabolic stability and pharmacokinetic profile *ex vivo* (Fig. 2, Lower right).

In summary, the structural optimization of the small molecules targeting the well-studied pathways significantly increases the potencies, selectively, tissue specificity and pharmacokinetic properties. Their long-term efficacy and safety in human subjects warrant further validation. Since NASH is driven by rewired metabolic pathways in a systemic way, it is reasonable to propose that these systemic pathways are the root causes of NASH, and thus can be normalized (Section 3).

## 3. The novel NASH targets regulate mitochondrial function and energy expenditure

Patients with NASH have the high probability of carrying other metabolic comorbidities, which means that targeting the pathogenic processes will restore the homeostasis of multiple organs and tissues. It may be essential for resolution of NASH. Therefore, besides the direct

intervention on the inflammation, steatosis and fibrosis pathways in liver, the emerging novel drug targets have been genetically verified. They don't have an apparently direct link with these dysfunctions in liver, yet their dysfunction significantly contributes to NASH progression. The efficacy of their small molecule modulators has been confirmed in preclinical models. The small molecules increase the systemic energy expenditure in a controllable way, or perturb the novel cellular pathways and eventually resolve inflammation, fibrosis and steatosis during NASH progression. The advantages and potential shortcomings will be carefully discussed.

Global increase in energy expenditure can lead to concomitant improvement of liver function, and thus the resolution of NASH. Furthermore, this systemic treatment is also superior to the previous therapies that simply increase insulin sensitivity to improve systemic metabolism, as these therapies didn't exhibit obvious improvement in liver pathohistology [69]. New strategies integrate both paradigms and have synergistic effects. For instance, the aldolase inhibitor Aldometanib targets both liver and skeletal muscles in murine NASH model to mimic the exposure of mice to low blood glucose level. Aldometanib activates lysosomal AMPK by preventing the binding of fructose biphosphate to aldolase and blocking its downstream pathways [70]. AMPK activation in multiple organs simultaneously suppresses inflammation, fibrosis and hepatic steatosis in murine NASH model (Fig. 3A) [70].

### 3.1. New mitochondrial uncouplers mediate thermogenesis and attenuate NASH

Dysfunctional mitochondria are closely associated with pathogenesis of NASH and thus mitochondria have emerged as promising therapeutic targets (Fig. 3B). Mitochondrial uncouplers are the small molecules that induce $H^+$ leak and generate ion current ($I_H$) across the inner membrane of mitochondria, and thus elicit thermogenesis [71]. They effectively elevate energy expenditure *in vivo*. Furthermore, reduction of excessive mitochondrial reactive oxygen species (ROS) can be mediated by mitochondrial uncouplers and it protects the cells from damage [72,73].

Nitazoxanide, the FDA-approved antiparasitic compound exhibits uncoupling activity and is tested in clinical trial for treatment of NASH-associated fibrosis [74]. SHC517, the uncoupler also significantly attenuates NASH, especially improves fibrosis, inflammation and serum markers of liver damage without obvious toxicity in a streptozotocin (STZ)-induced mouse model of NASH (diamine derivative, SHC517, Fig. 3C) [75]. Nevertheless, its pharmacokinetic properties are suboptimal, due to short half-life and low circulating concentration in the plasma of mice. Structural optimization substitutes amine group of diamine derivative with hydroxyl group, and it improves aqueous solubility and pharmacokinetic properties. Secondly, pentafluorination on aromatic ring yields the final optimized compound, SHM115 with optimal bioavailability and potent mitochondrial uncoupling activity [76]. It increases the energy expenditure in mice fed with HFD and a high-sugar Western diet (Fig. 3C).

The concern of mitochondrial uncouplers, specifically 2,4-dinitrophenol (DNP) and its derivatives, is their substantial clinical toxicity. It can be solved by controlled-release oral formulation of the therapeutics. For instance, the formulation of DNP with sustained systemic release function, which is called CRMP (controlled-release mitochondrial protonophore), directly delivered the therapeutics to liver and thus effectively ameliorated NASH symptoms in mice fed with a methionine/choline–deficient diet (MCD). The minimal effective dose for CRMP is 10-fold lower than DNP alone does. In contrast, the adverse effects and toxicity, such as disturbed liver function and elevated blood urea nitrogen, and creatinine have been minimal in rats treated with formulated DNP [77]. Thus, this study in *Science* is a proof-of-concept endeavor, and it indicates enhancing $H^+$ leak across mitochondria can potentially normalize the systemic energy homeostasis and resolve NASH through elevating energy expenditure in liver.

Besides traditional mitochondrial uncouplers, sorafenib (the Raf inhibitor to treat HCC) used at low dose is unraveled as a novel mitochondrial uncoupler. It elevates the intracellular AMP/ATP ratio and activates AMPK signaling to prevent the progression of NASH in primates and mice [78]. Nevertheless, cautions should be taken into account for administration of sorafenib, as it also binds to some off-targets, thereby generating potential side effects. For instance, sorafenib induces cellular ferroptosis and depletes intracellular glutathione (GSH), which are independent of its Raf kinase inhibitory effect [79–81]. These side effects are proposed to be mediated by suppression of cystine-glutamate antiporter-xCT [79]. As sorafenib activates ferroptosis in HCC [82], and ferroptosis is involved in the progression of NAFLD [83], the side effects of sorafenib should be rigorously analyzed.

The above canonical uncouplers are designed based on protein-independent protonophoric mechanism, and it confers them the protonophoric activity to affect mitochondrial membranes in certain cell types. The *in silico* docking and molecular dynamics simulations fuel the design of new mitochondrial uncouplers by directly activating ADP/ATP carrier (AAC, also called adenine nucleotide translocase) and UCP1. It may circumvent the nonspecific increase in $H^+$ conductance across membranes, which elicits toxicity [84,85]. The study in *Nature* has elucidated that the binding sites for uncouplers (protonophores) and long-chain fatty acids in AAC were overlapped with the ADP/ATP binding sites. The design of more specific AAC activators to increase $I_H$ and enhance thermogenesis may be a promising direction (Fig. 3C) [86].

In summary, the resolution of the AAC structure significantly deepens the understanding of the mechanism and design of mitochondrial uncouplers: First, it demonstrates that $H^+$ leak, and thermogenesis initiated by direct binding of uncouplers with AAC. Thus, it challenges the stereotyped view that mitochondrial uncouplers induce $H^+$ leak indiscriminately across the biological membranes, which is independent of proteins; Secondly, these studies provide structural insights into designing AAC modulators to combat NASH: AAC exchanges ADP and ATP through the mitochondrial inner membrane via an antiport mechanism, and this process is critical for coupled respiration [87]. Its inhibition protects the mice from NAFLD and obesity [88]. Taken together, the small molecules that divert AAC from coupling respiration to increase $H^+$ leak may effectively attenuate NASH in a systemic way.

Up to now, most of the metabolic benefits of mitochondrial uncouplers have been observed in rodent models. The latest phase IIb trial in human subjects with NAFLD and high BMI revealed that the small molecule drug HU6 could be metabolized in liver and converted to DNP. It exhibits well-tolerance and safety, as well as promising efficacy [89].

### 3.2. Targeting mitochondrial proteases normalizes energy homeostasis

The primary function of hepatic mitochondria is to produce energy, especially ATP synthesis via oxidation of pyruvate, fatty acids and amino acids. Numerous circulating and intrahepatic molecules finely tune the flux of metabolites in mitochondria to coordinate ATP synthesis and oxidative phosphorylation [90]. Dysfunction of mitochondria induced by excessive nutritional and inflammatory stimuli results in defective mitophagy. Mitophagy is a specific type of autophagy that eliminates the damaged or excess mitochondria, while it is reduced during NASH progression [91]. Defective mitophagy stimulates the TNFα production (Miz1-PRDX6 axis) by hepatocytes and macrophages, and triggers the subsequent inflammatory cascades [92]. Moreover, it leads to the maladaptation of mitochondria, and reduces oxidative capacity and increases lipid accumulation during the progression of NASH (Fig. 3B). Restoration of mitochondrial function by small molecules attenuates oxidative stress, and mitophagy is thus normalized. For instance, natural products (flavones) lower reactive oxygen species [93], and prebiotics improve mitophagy to ameliorate NASH [94]. Nevertheless, their benefits are mediated by non-specific mechanisms.

Recent studies have indicated the function of mitochondrial protease systems in degradation of unfolded proteins under metabolic stress,

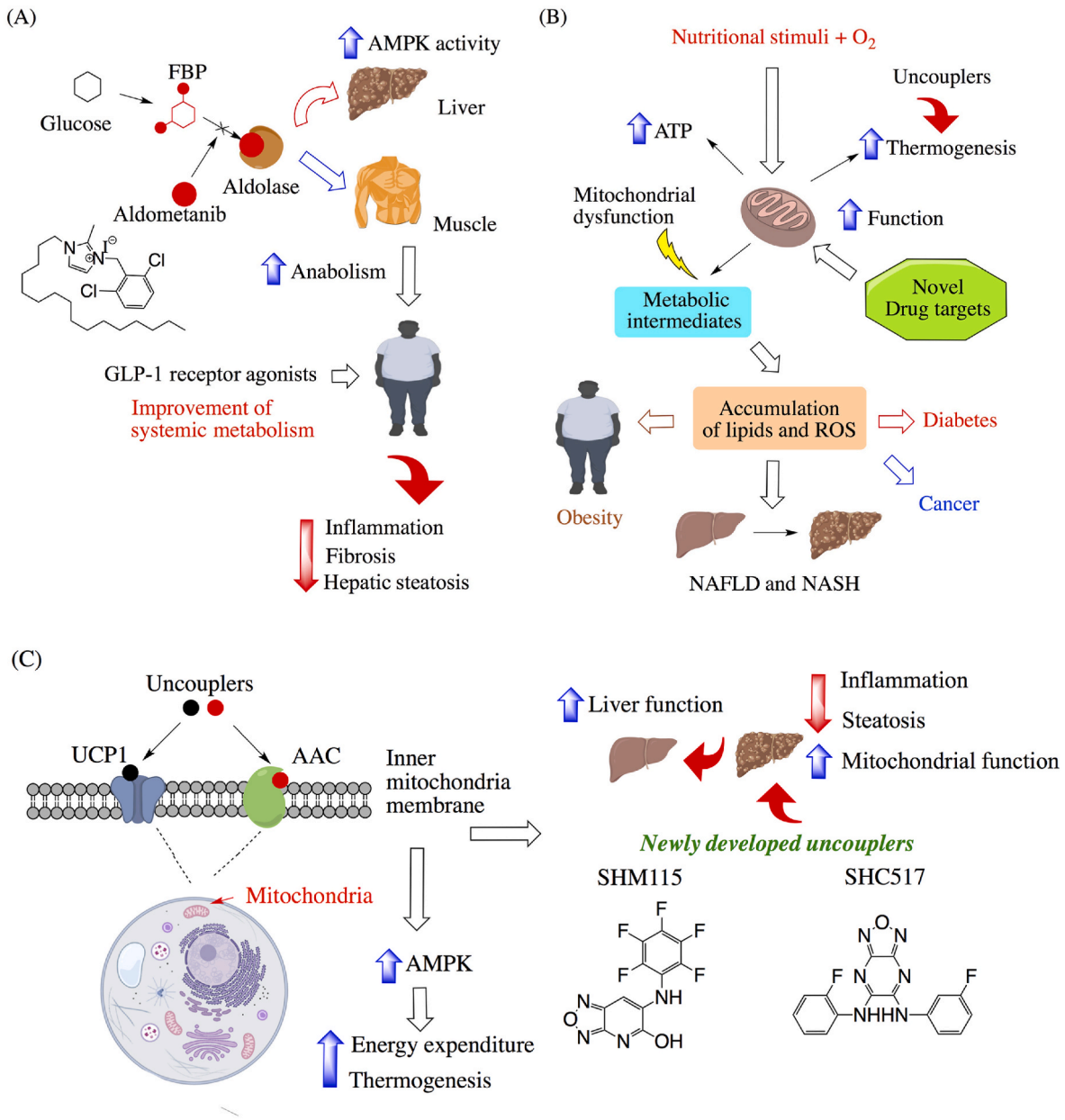


**Fig. 3.** Normalization of systemic metabolism resolves NASH. (A) Targeting the key pathways in systemic metabolism during NASH progression, e.g. GLP-1 and aldolase effectively ameliorate NASH in rodents and human subjects; (B) Dysfunction of mitochondria leads to aberrant accumulation of lipids and ROS. They trigger dysregulation in whole body metabolic homeostasis, metabolic disorders and NASH. (C) Elevation of thermogenesis in mitochondria with mitochondrial uncouplers by activating both UCP1 and AAC increases energy expenditure and alleviates NASH by normalization of systemic metabolism.

thereby serving as a quality control system to govern the energy homeostasis and progression of NASH. They serve as potential drug targets (see Fig. 4A). Proteases belong to mitochondrial protease systems, such as caseinolytic protease (ClpP) and ClpXP (complex formed by ClpP-AAA ATPase chaperone, ClpX). They degrade misfolded proteins and thus contribute to maintenance of mitochondrial proteostasis (protein homeostasis) [95]. Thus, dysfunction of proteostasis contributes to mitochondria dysfunction. Notably, two independent studies both unraveled the novel regulatory mechanisms of ClpP and ClpXP, and they may be perturbed by small molecules to regulate mitophagy, homeostasis of oxidative stress and inflammasome activation. Deficiency in ClpP induces mitochondrial dysfunction and promotes NASH progression in murine models, while pharmacological activation, such as the acyldepsipeptide, A54556A activates ClpP and therefore dampens steatohepatitis in C57BL/6J mice (Fig. 4B) [96].

Besides the direct activation of mitochondrial proteases to maintain the mitochondrial homeostasis, the stability of key factors that regulate mitophagy also critically contributes to NASH progression mediated by post-translational modification. For instance, the mortality factor 4-like protein 1 (MRG-15) is the chromatin remodeler and it interacts with nuclear receptor LRH-1. Then MRG-15 is recruited to genomic loci in proximity to lipid synthesis genes, which is facilitated by LRH-1. MRG-15 thus directly activates the transcription of genes in *de novo* lipogenesis [97]. In addition, a recent study has indicated that hepatic MRG-15 is upregulated in patients with NASH and aggravates NASH progression. Consistently, MRG-15 knock-in mice exhibited much exaggerated NASH progression, while hepatic depletion of MRG-15 with adeno associated virus (AAV)-sgRNA alleviated hepatomegaly and steatosis, fibrosis and inflammation [98]. TNFα and IL-6 induces acetylation of MRG-15 and thus stabilizes MRG-15. MRG-15 interacts with mitochondrial Tu

translation elongation factor (TUFM, which modulates autophagy) and deacetylates TUFM at the K82 and K91 sites [99]. The deacetylation of TUFM accelerates its degradation mediated by the mitochondrial ClpXP protease system. It impairs mitophagy, increases oxidative stress and activates the NLRP3 inflammasome pathway [98].

The advantage of targeting mitochondrial protease system is that the proteases are druggable, with well-studied substrate-binding domains. Moreover, MRG-15 can also be blocked by FDA-approved drug argatroban (thrombin synthesis inhibitor for treating heparin-induced thrombocytopenia), and the treatment diminished steatosis in murine models [97]. The specific MRG-15 antagonists will serve as lead compounds for drug discovery (Fig. 4C).

Nevertheless, these studies are focused on murine models, in which their anti-fibrotic activities have not been rigorously assessed so far. Furthermore, whether restoration of mitochondrial homeostasis is sufficient to reverse NASH progression in patients warrants further validation.

### 3.3. Stabilization of RNA helicase DEAD-box protein 5 by small molecules alleviates NASH

Stabilization of RNA structure and expression level are essential for energy homeostasis in liver and has emerged as novel therapeutic strategies for NASH treatment. DEAD-box protein 5 (DDX5) is an ATP-dependent RNA helicase that regulates the stability and processing of RNA, DNA [100]. In addition, it is a co-regulator of different transcription factors and regulates transcription of multiple genes [101]. It has a broad range of (patho)physiological activities, and it is highly expressed in malignant tumors and contributes to the production of circular RNA [102]. It also enables translation of transcription factor STAT1 to evoke interferon response in hepatitis B virus-replicating hepatocytes [103].

DDX5 is pivotal for the homeostasis of liver, as it interacts with E2F transcription factor 1 (E2F1) to induce the expression of Atg4B, and thereby triggering mitophagy to stimulate the regeneration of liver in aged mice [104]. DDX5 expression is downregulated in liver from patients and rodents with NASH [105]. The protective effect of DDX5 is mediated by recruiting the tuberous sclerosis complex (TSC)1/2 complex and then it negatively regulates mTORC1 activity, thereby attenuating the aberrantly activated lipogenesis process and NLRP3-mediated inflammation [105]. Therefore, small molecules that enhance the stability, or increase the expression level of DDX5 have the potential to trigger mitophagy and thus to dampen the hepatic steatosis and inflammation simultaneously to resolve NASH. For instance, the natural product hyperforcinol K from hypericum medicinal plant directly interacts with DDX5 to block the ubiquitination of DDX5, which is mediated by tripartite motif containing 5 (TRIM5), the ubiquitin ligase (E3). Increased stability of DDX5 significantly mitigates the hepatic steatosis and inflammation in murine models [105].

The study on DDX5 stabilization in ameliorating NASH has indicated new trends and insights in NASH therapies [1]: Activation of DDX5 integrates the anti-steatosis and anti-inflammatory pathways simultaneously to ameliorate NASH [2]; The mechanism of anti-NASH activity of hyperforcinol K is novel, as it can recapitulate the effects of direct activation of DDX5. It is also unique as potent DDX5 activators/agonists have not been reported so far [3]; DDX5 stimulates autophagy and blocks mTORC1 activities, and contributes to the suppression of HCC [106]. Whether activation or stabilization of DDX5 can block or delay the NASH-HCC transition warrants further investigation.

Targeting DDX5 still has some challenges. First of all, the resolution of NASH symptoms mediated by DDX has so far been documented in rodent models. Its clinical relevance in patients with NASH requires

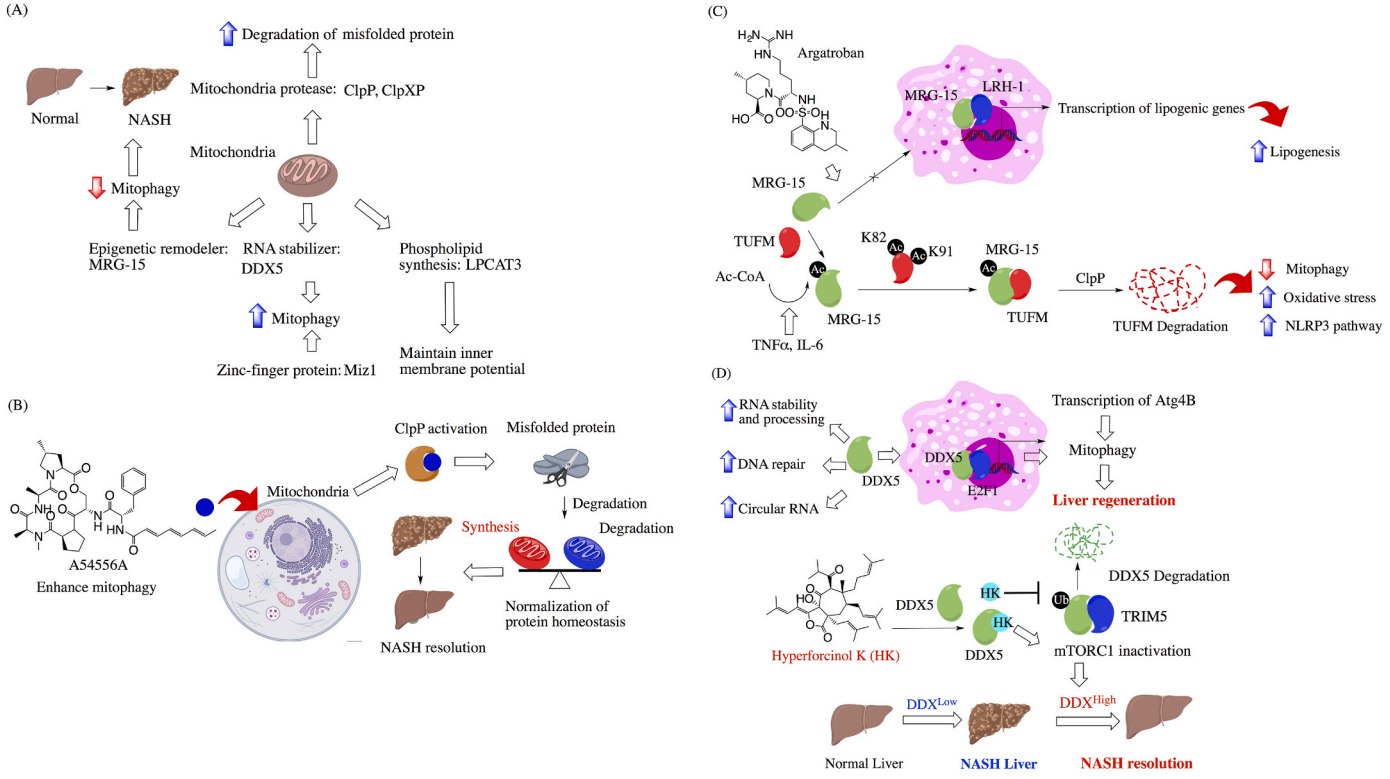


**Fig. 4.** Illustration of novel genes that regulate mitochondrial function, NASH progression and their small molecular modulators. (A) The recently identified genes modulate mitochondrial function and thus NASH progression. (B) A54556A activates ClpP and results in normalization of mitochondrial protein homeostasis to resolve NASH; (C) MRG-15 interacts with LRH-1 in nucleus and triggers the transcription of lipogenic genes, which is blocked by Argatroban. Acetylation of MRG-15 by inflammatory factors promotes the ubiquitination (Ub) of TUFM and its degradation by ClpP; (D) The overview of DDX's function in blocking NASH progression. DDX5 interacts with E2F1 to stimulate the transcription of Atg4B and leads to mitophagy. Hyperforcinol K inhibits the ubiquitination of DDX and thereby inactivating mTORC1 signaling pathways to suppress NASH progression.

rigorous validation. Analyzing the correlations of human SNP in DDX sequence, or its aberrant expression levels during NASH progression will be a prerequisite for conducting clinical studies.

Secondly, the high hydrophobicity of hyperforcinol K, and the difficulty in synthesis may pose significant challenges. The pharmacokinetic properties, long-term adverse effects and toxicity of hyperforcinol K and derivatives in human subjects remain unknown. The structure-activity relationship studies on hyperforcinol K to identify more potent analogs with optimal pharmacokinetic properties are anticipated. Moreover, the crystal structure of N-terminal region of DDX5 has been solved, thereby paving the way to design novel DDX5 agonists with more scaffolds (Fig. 4D) [107].

The recently identified and genetically validated genes that regulate NASH progression through the improvement of mitochondrial function are summarized in Table 2.

### 3.4. mTORC pathways and new drug targets: Dyrk1b

In Section 3.3, we described that DDX negatively regulates mTORC1 activity. Mechanistic target of rapamycin complex (mTORC) pathway is the principal pathway that regulates the cellular anabolism [110]. mTORC governs the lipid and protein synthesis, and aberrant activation of lipid synthesis significantly contributes to the development of metabolic liver diseases stimulated by lipogenesis. Direct activation of mTORC pathway elicits hepatic inflammation. Increased mTORC1 activation also impedes lipophagy and lipid droplet degradation [111]. On the other hand, mTOR pivotally inhibits AMPK and autophagy [112]. Regulation of mTOR activity by either genetic manipulation of upstream regulators, or by potent small molecules effectively activate autophagy and ameliorate the symptoms of NASH in murine models [113–116]. In summary, regulators in mTORC signaling pathway may serve as novel therapeutic targets for resolution of NASH.

For instance, tyrosine phosphorylation-regulated kinase 1b (Dyrk1b) directly activates mTORC2 in a kinase independent way to stimulate *de novo* lipogenesis specifically in hepatocytes of mice fed with high-calorie diet [117]. Silencing of Dyrk1b in liver with AAV protects mice from steatosis. Mechanistically, Dyrk1b activates Rictor, an obligate mTORC2 subunit and stimulates the autophosphorylation of mTORC2, which is independent of hepatic insulin signaling. In contrast, blockade of hepatic mTORC1 signaling pathway significantly alleviates steatosis, inflammation, and fibrosis in methionine choline-deficient (MCD) diet-fed mice [118]. On the other hand, hepatic TXNIP/VDUP1 (thioredoxin interacting protein) interacts with phosphorylated PRKAA (a catalytic subunit of AMP-activated protein kinase, AMPK) and inactivates mTORC1. Consistently, the well-established mTORC1 inhibitor rapamycin reduces lipid accumulation and inflammation *in vivo* when TXNIP is ablated [118]. Thus, utilization of the currently safe inhibitors of mTORC in treatment of NASH maybe an alternative option [119].

Nevertheless, mTORC pathway has the pleiotropic effect and is essential for the growth, proliferation and homeostasis of tissue and organs. Indiscriminative blockade of mTORC pathway by inhibitors may generate adverse effects, even toxicity *in vivo*. Liver-specific delivery of mTORC inhibitors using nanoparticles will enrich the drugs in liver and increase the potency, while lowering systemic toxicity.

### 3.5. Inhibitors for endoplasmic reticulum (ER) stress

Endoplasmic reticulum (ER) stress, which is featured with accumulation of misfolded/unfolded proteins in the ER lumen, triggers a myriad of signaling cascade, especially unfold protein response (UPR) to reinstate the homeostasis of ER [120]. Nevertheless, chronically unresolved ER stress promotes cell death and inflammation. ER stress is one of the hallmarks of metabolic complications and is required for NASH development, for instance via ATF4-SLC7A11 axis [121]. Chronic hepatic ER stress not only triggers cell death via activation of NLRP3 [122], but also drives lipogenesis and steatohepatitis via induction of caspase-2 expression and activation of site 1 protease [123]. Furthermore, nutrient and diet-induced ER stress are mediated by activation of inositol-requiring transmembrane kinase endoribonuclease-1α (IRE1α). IRE1α promotes the release of ceramide-enriched inflammatory extracellular vesicles by hepatocytes to aggravate NASH progression [124]. The role of IRE1 in mediating NASH progression has also been verified by an independent study, in which ER stress activates IRE1, and inhibition of IRE1 in murine NASH model blunts steatohepatitis and dampens the expression of insulin induced gene 2 (INSIG2) expression [125].

Blockade of aberrant and persistent ER stress has emerged as a promising strategy to simultaneously attenuate hepatic steatosis, inflammation and fibrosis. Genetic ablation of X-box binding protein-1 (Xbp1) in hepatic macrophages and hepatocytes reduces inflammation [126], and mitigates liver fibrosis via cGAS/STING/NLRP3 signaling [127]. Among the key regulators of ER stress, Xbp1, ATF6 and CHOP are transcription factors that are challenging to target; In contrast, IRE1α contains a kinase domain and has RNAse activity. It has the potential to design inhibitors to suppress its kinase-RNAse activities. Consistently, inhibition of IRE1α significantly attenuates hepatic inflammation and lipid accumulation [128]. For instance, IRE1α inhibitor 4μ8C ameliorates inflammation, steatosis and liver injury in mice with NASH [124].

Initially, IRE1α inhibitors were designed to eradicate cancers. Nevertheless, these promising *in vitro* anticancer results shed insights into design of NASH therapeutics. The well-optimized IRE1α inhibitors can be repurposed to treat NASH, which provides new options compared to the previous anti-inflammatory and anti-steatosis strategies. Among the IRE1α inhibitors, the crystal structure of 4-quinazoline-1,3-dimethyl-pyrazole ligand bound with human IRE1α has been solved. Rational design afforded the compound as the most potent lead

**Table 2**
List of the recently validated genes since 2022 that regulate NASH progression through mitochondrial function.

| Gene | Full name | Mechanism of action in NASH progression | Reference |
|---|---|---|---|
| ClpP | Caseinolytic protease P | Activation of ClpP reduces mitochondrial dysfunction to ameliorate NASH | [96] |
| DDX | DEAD-box protein 5 | Recruits the (TSC)1/2 complex and negatively regulates mTORC1 activity, promotes mitophagy to inhibit NASH | [105] |
| EIF5A | Eukaryotic initiation factor 5A | Hypusination of EIF5A partially restores protein synthesis and mitochondrial function in NASH, and prevents NASH progression | [108] |
| LPCAT3 | Lysophosphatidylcholine acyltransferase 3 | Loss of Lpcat3 in mouse liver increases inner mitochondrial membrane phospholipid saturation and enhances reactive oxygen species production | [109] |
| MRG15 | Mortality factor 4-like protein 1 | Increased acetylation in MRG15 facilitates the interaction and deacetylation of the mitochondrial TUFM to impairs mitophagy | [98] |
| TNFα/ Miz1 | Zinc-finger protein 1 | Loss of hepatocyte Miz1 results in PRDX6-mediated inhibition of mitophagy, increases dysfunctional mitochondria in hepatocytes | [92] |

compound with extremely high inhibitory potency (IRE1α-TR-FRET $IC_{50}$ = 4.4 nM) (Fig. 5A) [129]. In addition to translational perspective, these IRE1α inhibitors can also serve as specific tools to perturb the function of IRE1α and ER stress, which offers a new effective approach in deciphering the function of ER stress during NASH progression.

In summary, these recently validated drug targets regulate the progression of NASH through novel mechanisms to normalize the cellular homeostasis and systemic metabolism. As a great portion of them are still in preclinical investigation, the long-term efficacy and adverse effects are the major concern.

## 4. The secreted proteins-receptor signaling regulates NASH progression

### 4.1. Secreted proteins greatly affect NASH progression

Cross-talks between intrahepatic cells and other organs mediated by secreted proteins impact the pathogenesis of NASH (Fig. 6A) [130,131]. Notably, livers from rodents and human secrete peptides and proteins into circulation to integrate and reprogram the signaling pathways of multiple tissues or organs, and regulate the progression of NASH. Thus, there are two principal therapeutic approaches:

The first is that recombinant secreted proteins with optimal stability *in vivo* can treat NASH through systemic normalization of metabolic homeostasis. For instance, FGF21 holds the great promise in NASH resolution [132]. Long-acting Fc-FGF21 fusion protein, efruxifermin significantly reduced hepatic steatosis in patients with NASH (Fig. 6A) [133]. Notably, the hepatic fat fraction of the patients was dramatically decreased. In addition, the latest randomized, controlled phase IIb trial of the FGF21 analogue Pegozafermin on patients with NASH achieved 37 % resolution of NASH and improvement of fibrosis, thereby supporting the phase III development. The safety of Pegozefermin has also been assessed, with nausea and diarrhea as the most common adverse effects. These side effects are generally manageable [134].

On the other hand, synthetic small molecules can directly interact with the receptors of secreted proteins, and resolve NASH by recapitulating the function of secreted proteins in multiple tissues simultaneously [135].

Besides the secreted proteins from liver, adipose tissue and muscle, neuropeptides in central nervous system (CNS) also modulate the NASH progression by activating AMPK-mediated signaling transduction in liver. Hypothalamic neurons secrete kisspeptin, the neuropeptide and it binds to its receptor in hepatocytes to activate AMPK signaling pathway. It consequently dampens lipogenesis and enhances oxidation of lipids [136]. Agonists activate hepatic kisspeptin receptor and block the NAFLD-NASH transition, leading to alleviation of NASH in murine models [136]. The kisspeptin receptor agonists are currently under clinical investigation to treat reproductive disorders in human, thereby holding promise in their reutilization in treatment of NASH [137].

### 4.2. Adiponectin receptor agonists mimic the function of hormones in vivo

Administration of either GLP-1 receptor agonist semaglutide alone, or dual GLP-1/GLP-2 receptor agonist, GLP1/2-Fc fusion protein resolves NASH in patients and rodents [25,138]. Moreover, semaglutide combined with FXR agonist cilofexor, and the acetyl-coenzyme A carboxylase inhibitor firsocostat improve liver steatosis and fibrosis in patients with NASH [139]. These successes indicate that modulation of the function of novel hormone receptors by small molecules can recapitulate the above-mentioned function of hormones to regulate metabolic homeostasis.

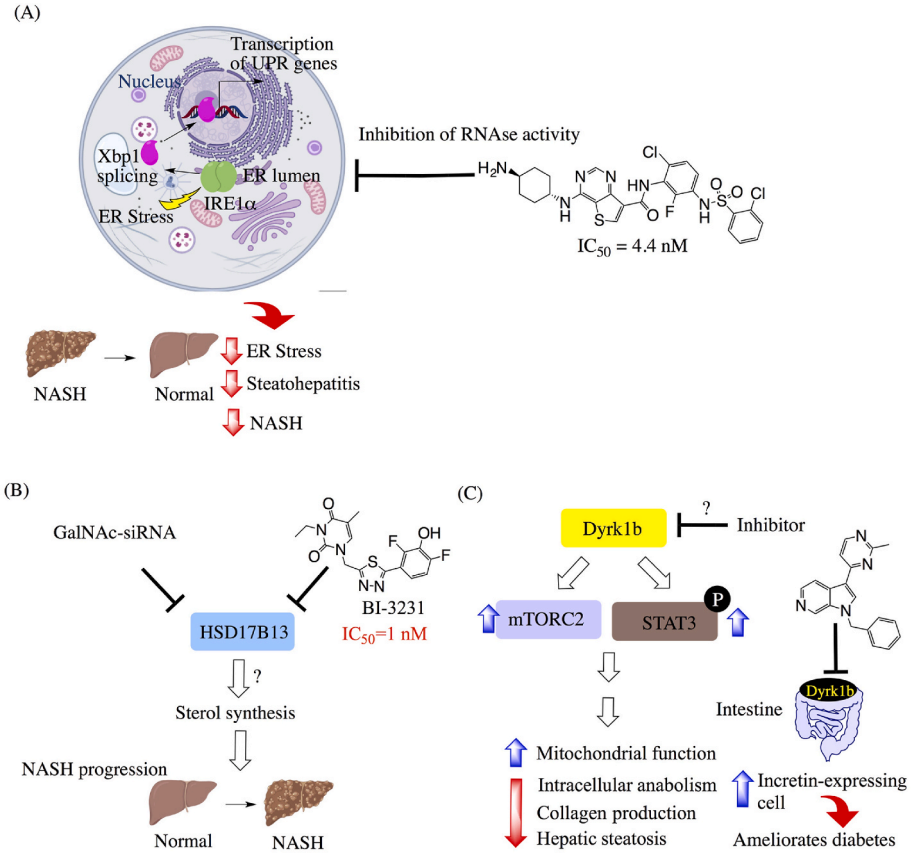


**Fig. 5.** Novel drug targets that significantly regulate NASH progression. (A) IRE1α inhibitorsblock ER stress to alleviate NASH progression; (B) Highly potent HSD17B13 inhibitors block the function of HSD17B13, which is freely available via the opnMe platform; (C) Dyrk1b inhibitors suppress mTORC2 function and thus lipogenesis, as well as increase incretin-expressing cells in intestine.

Besides incretin (e.g. GIP and GLP-1), another prominent example is adiponectin. Adiponectin is encoded by the *apM1* gene and is secreted by adipose tissue. It binds to its receptors (AdipoR1 and AdipoR2) to mediate glucose uptake, and fatty acid oxidation, to enhance insulin signaling transduction and diminish inflammation [140]. Down-regulation of adiponectin and its receptors were observed in patients with obesity and type II diabetes [141]. In contrast, higher adiponectin level induced by PPAR-γ agonists is associated with improved steatohepatitis in patients with NASH [142]. Adiponectin/AdipoR signaling also prevents mitochondrial dysfunction [143].

The small molecule adiponectin receptor agonist, AdipoRon integrates energy expenditure with anti-inflammatory, steatosis and fibrotic activities. AdipoRon binds to both AdipoR1 and AdipoR2, and recapitulates the physiological activity of adiponectin in liver and muscle by ameliorating insulin resistance and glucose intolerance in mice fed with HFD [144]. Furthermore, the optimized AdipoRon derivative, Q7 has exhibited higher potency compared with AdipoRon in reducing fibrosis, inflammation, steatosis and liver injury in murine NASH model (high-fat, high-cholesterol, and high-fructose diet) (Fig. 6B) [145].

Besides AdipoRon derivatives, oligopeptides also reveal promising anti-NASH potencies. For instance, tripeptides from soybean activate AdipoR1 and their anti-NASH potencies are expected to be investigated in the future [146]. Moreover, AdipoR1/AdipoR2 dual agonist JT003 activates both AdipoR1/AdipoR2, as well as AMPK, PPARα, and PI3K-Akt pathways. It further attenuates ER stress and mitochondrial dysfunction in murine model with HFD-induced steatohepatitis and $CCl_4$-induced fibrosis (Fig. 6B) [135]. More importantly, its long half-life in circulation and favorable pharmacokinetic properties confer JT003 as a promising hit for future optimization. Combination of JT003 with inhibitors that block the lipogenesis and fibrosis induced by degraded extracellular matrix, is able to synergistically enhance mitochondrial function via AMPK pathway and resolve NASH in murine model [147].

The uncertainty herein is to which extent JT003 can recapitulate the function of adiponectin *in vivo*. Moreover, whether it has off-target effects by binding to other protein targets needs to be systemically evaluated. Regarding to anti-fibrotic activity, although JT003 mitigates $CCl_4$-induced fibrosis in the murine model, whether it can induce long-time anti-fibrotic activity under the context of worsening fibrosis in NASH patients awaits investigation.

### 4.3. Retinol binding protein 4 (RBP4) antagonists normalize liver function

Retinol binding proteins are synthesized and secreted by liver and specifically transport retinol in the circulation. They form 1:1 complex with the serum transthyretin [148]. Among the retinol binding proteins, RBP4 is proposed as a hepatic cytokine and its aberrant expression contributes to multiple metabolic disorders. Higher hepatic RBP4 levels are correlated with pathohistological features of NASH patients [149]. Its transcription is stimulated by activated STAT3 in hepatocytes upon exposure to TNFα [150]. RBP4 promotes M1 polarization of hepatic Kupffer cells via activation of NOX2/NF-κB/TNFα pathway. Suppression of RBP4 function, such as silencing RBP4 by RNA oligonucleotide in adipose tissue of mice fed with HFD alleviates hepatic steatosis and normalizes liver function [151].

Protein Data Bank (PDB) high-resolution X-ray crystal structure is able to reflect the interaction between ligand and RBP4. RBP4 is capable of binding lipid ligands other than retinoids. Thus, the optimized bicyclic antagonist, BPN-14136 is designed based on PDB data and is a non-retinoic acid. It exhibits excellent *in vitro* RBP4 binding property, functioning antagonistic activity and good *in vivo* pharmacokinetic properties [152]. Furthermore, N-benzyl imidazole derivatives (NID) have been structurally optimized and effectively disrupted RBP4-transthyretin interaction, along with low clearance and good half-lives in rodents (Fig. 6C) [153].

RBP4 antagonists are currently under clinical trial to evaluate their efficacies in treating Stargardt disease (STGD, an inherited disorder that usually causes vision loss in childhood or adolescence) [154]. The long-term efficacy, toxicity and pharmacokinetic property will definitely guide the application of RBP4 antagonists in treating NASH. Nevertheless, RBP4 is a liver-specific secreted cytokine, thus its binding proteins and downstream signaling pathway that mediate metabolic diseases in multiple organs or tissues are not well deciphered. The adverse effects of chronic blockade of RBP4 function *in vivo* should be assessed rigorously in the future.

The recently identified and rigorously validated secreted proteins that regulate NASH progression are summarized in Table 3.

It should be cautious of the oversimplified model to categorize the function of secreted proteins as "NASH-promoting" or "NASH-blocking". For instance, distinct subsets of liver cells secret peptides that may have mutually opposite functions, which is exemplified by osteopontin. Osteopontin secreted by liver macrophages protects mice from NASH by induction of arginase-2 and subsequent enhancement of fatty acid oxidation [155], whereas the overexpression of osteopontin in hepatic stellate cells, and treatment of mice with osteopontin are able to elevate the expression of COL1 and promote liver fibrosis [156,157]. In summary, it is critical to delineate the cell subtypes in liver by which they secret peptides/peptides, or they express the receptors for secreted proteins to regulate NASH progression.

## 5. Newly identified NASH targets

Here we will discuss the mechanisms of the newly identified enzymes that regulate intracellular metabolic homeostasis during NASH progression, and the development of their small molecule modulators. Unlike the above drug targets with many delineated mechanisms and well-developed modulators, the function of the newly identified enzymes has only been appreciated.

### 5.1. The first selective HSD17B13 inhibitor: new probes, new horizon

Small molecule modulators are tools for manipulation of new NASH targets to regulate energy homeostasis, which is complementary to the genetic validation of their function *in vivo*. For instance, hydroxysteroid 17β-dehydrogenase 13 (HSD17B13) is a lipid-droplet associated member of the family of 17β-hydroxysteroid dehydrogenases (HSD17B), the oxidoreductases that catalyze the last steps of steroid biosynthesis [162]. It is expressed specifically in hepatocytes, with retinol and estradiol as the known substrates [163]. Genome-wide association studies (GWAS) have indicated its splice variant (rs72613567: TA) encoded a loss-of-function truncated variant, which is associated with a reduced risk of nonalcoholic steatohepatitis, but not steatosis in human liver samples. Another causative splice variant, rs72613567 is functionally associated with NAFLD histology and protection from liver fibrosis [163–165]. In addition, SNP rs72613567:TA in patients with NASH is associated with a lower risk for developing HCC, which suggests it may regulate advanced stage of NASH and NASH-HCC transition [166].

Overexpression of HSD17B13 in murine livers induced a fatty liver phenotype, with augmented lipogenesis [167]. HSD17B13 also promotes NASH pathogenesis through stimulating pyrimidine catabolism, while silencing of HSD17B13 protects mice from liver fibrosis via reduction of pyrimidine catabolism [168]. Administration of GalNAc-conjugated siRNA in a proof-of-concept trial against HSD17B13 exhibited good tolerance and effectively reduced ALT and AST levels in patients with NASH [16]. Clinical trial based on RNA interference (RNAi) strategy has been launched recently [37]. Nevertheless, the efficacy of RNAi strategy is complicated by off-target effects and thus toxicity [169]. Therefore, small molecules may be an alternative option.

Collectively, development of small molecule inhibitors for HSD17B13 is the prerequisite to ameliorate the off-target effects elicited by RNAi. Recent pilot high-throughput screen has identified BI-3231,

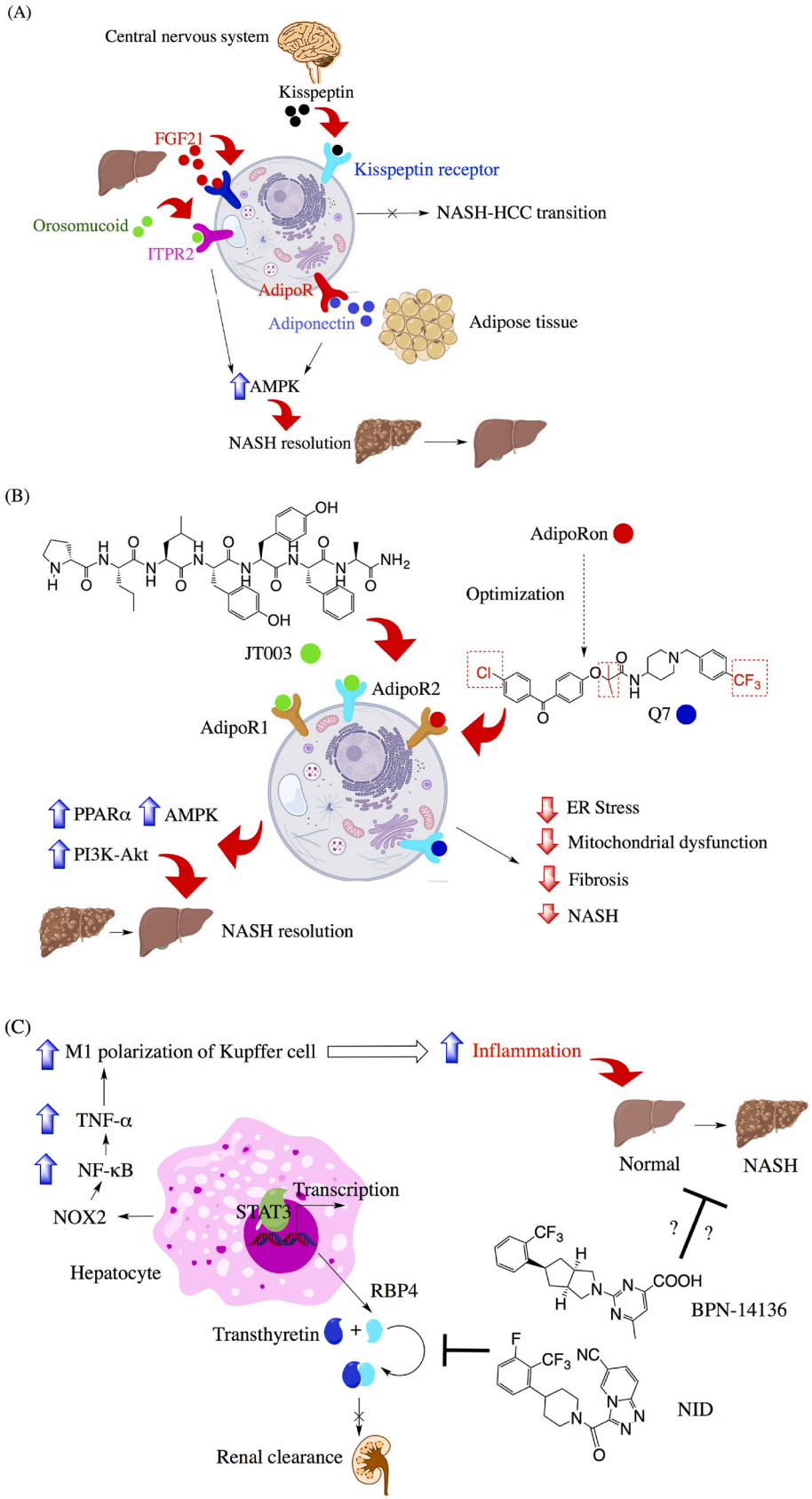


**Fig. 6.** Secreted proteins from multiple organs regulate the progression of NASH and the therapeutics that target the signaling transduction to resolve NASH. (A) Overview of representative secreted proteins which are recently validated to resolve NASH; (B) Adiponectin receptor agonists JT003 (peptide), AdipoRon and optimized derivative Q7 bind to and activate adipoR1 and adipoR2 simultaneously to resolve NASH; (C) Mechanism of RBP4 *in vivo* and blockade of its signaling transduction by its antagonist BPN-14136. RBP4 antagonists STG-001 and tinlarebant are currently in clinical trials.

**Table 3**
List of the recently validated secreted proteins since 2022 that regulate NASH progression.

| Gene | Full name | Mechanism of action in NASH progression | Reference |
|---|---|---|---|
| ARSA | Arylsulfatase A | A liver-secreted lysosomal protein that degrades the glycosphingolipid sulfatides to galactosylceramides and inhibits insulin resistance | [158] |
| GREM1 | Gremlin 1 | A secreted antagonist that blocks BMP4 signaling to promote hepatic senescence and NASH | [159] |
| KISS1-KISS1R | Kisspeptin 1 receptor | Loss of hepatic KISS1R signaling increases hepatic steatosis and the progression to NASH | [136] |
| OPN (SPP1) | Osteopontin | Macrophage secretes OPN and upregulates oncostatin-M (ORM). ORM increases arginase 2 through STAT3 signaling to enhance fatty acid oxidation | [155] |
| ORM2 | Orosomucoid 2 | ORM2 binds to inositol 1, 4, 5-trisphosphate receptor type 2 to activate AMPK | [160] |
| WISP1 | WNT1-inducible signaling pathway protein 1 | Inhibition of WISP1 blocks the progression of existing liver fibrosis in NASH models | [161] |

the small molecule that displays extremely high potency against human HSD17β13 ($IC_{50}$ = 1 nM, Ki = 0.7 nM, Fig. 5B) and the majority of BI-3231 is enriched in liver with optimal pharmacokinetic properties [170].

BI-3231, the first selective HSD17B13 inhibitor shows the paradigm of how medicinal chemistry accelerates the study in NASH treatment. Nevertheless, its long-term potency in amelioration of NASH in patients awaits further validation, especially it is unknown whether the pharmacological inhibition can recapitulate the loss-of-function effects of HSD17B13 variant *in vivo*. Secondly, human genetics study indicates that rs72613567 polymorphism only protects the limited subgroup of patients from steatohepatitis and fibrosis [171]. Thus, the generality of HSD17β13 inhibition in patients with wide and heterogeneous spectrums of genetic traits needs to be validated. Lastly, the physiologically relevant substrates of HSD17β13 are not delineated. Thus, identification of its lipid substrates is essential for elucidation of its function *in vivo*.

### 5.2. The development of Dyrk1b inhibitor

The kinase Dyrk1b is upregulated in patients with metabolic syndrome in Egyptian cohort [172]. Besides its key function in ribosomal DNA double-strand breaks repair, Dyrk1b activates mTORC2 and promotes lipogenesis in a kinase-independent way (Section 3.4) [173]. Moreover, it mediates collagen production in hepatic stellate cells [174]. Dyrk1b in cardiac cells directly binds to STAT3 and increases its phosphorylation and nuclear translocation, thereby downregulating the PGC-1α expression and impairing mitochondrial function [175].

Dyrk1b inhibitors may effectively treat NASH as they address multiple root causes in NASH, without direct participating into the previously well studied pathways: 1) Excessive intracellular anabolism; 2) Collagen production; 3) Hepatic steatosis. The small molecule inhibitors may improve hepatic mitochondrial function and normalize systemic metabolic homeostasis. In support of this, they normalize the cardiac mitochondrial bioenergetics in mice with cardiac hypertrophy [175]. Dyrk1b inhibitors also have metabolic implications as they increase the number of incretin-expressing (GLP-1 and GIP) cells *in vivo*, through which they significantly increase the glucose tolerance and insulin sensitivity of diabetic mice [176].

The development of Dyrk1b inhibitors has been accelerated by set-up of a lead compound optimization pipeline. It successfully combines with computational simulation to identify specific Dyrk1b inhibitors [177]. There are still some caveats in their application in treatment of NASH, as current studies have only demonstrated its efficacy in improvement of glucose metabolism in murine models. The normalization of liver function by Dyrk1b inhibitors *in vivo* requires rigorous validation. Here shown are the potent Dyrk1b inhibitors in Fig. 5C.

The recently identified and rigorously validated genes that regulate NASH progression with other novel functions are summarized in Table 4.

## 6. Discuss and future direction

Novel drug targets intervene with previously underappreciated pathways and thus provide new horizons for treatment of NASH. They are able to integrate the multifaceted pathways that drive NASH pathogenesis and their modulation by small molecules belongs to the methods that tackle with "root cause" in Section 1.2. In addition, the animal models for evaluation of NASH therapeutics and the stages when the interventions initiate critically determine the success of NASH treatment. Fibrosis, cirrhosis and NASH-HCC transition are the challenging questions that need to be addressed in the future. Another future direction is to compare the efficacy and safety of the small molecues in multiple NASH models to unravel the genetic traits that determine the responses and the timing of dosage. Here we will discuss the perspectives in identification of NASH drug targets and optimization of current therapeutics.

### 6.1. Further optimization of current therapeutics to enhance efficacy

Current medicinal chemistry, combined with structural biology, has successfully improved the efficacy, pharmacokinetic properties and safety of NASH therapeutics. Some significant challenges that hamper the efficacy of the therapeutics still exist, and they can be addressed by multidisciplinary approaches. First of all, some pathogenic genes regulate NASH progression independent of ligands or their canonical function [117]. Secondly, the conventional pharmacological inhibition of pathogenic proteins elicits the activation of compensatory pathway [192]. These limitation prompts the creation of innovative proteolysis-targeting chimeras (PROTAC) technology that directly degrades the target protein via proteasome, or lysosome-dependent way [193,194]. PROTAC is potent and specific *in vivo* at much lower doses than conventional inhibitors and is currently under clinical investigation [195]. Modification of current NASH therapeutics into PROTAC can effectively enhance their efficacy and reduce adverse effects.

Another common problem in the therapeutics against NASH is the suboptimal liver-specific distribution. Nanoparticles that selectively deliver the therapeutics into liver have been extensively reviewed, as well as the cell subtype specific delivery of therapeutics into liver [196–198]. The combination of nanoparticle with drugs that reverse liver sinusoid capillarization is able to increase their efficacy and lower the adverse effects of treating NASH, especially it solves the most challenging problem: anti-fibrosis. For instance, pretreatment of liver cells with soluble guanylate cyclase stimulator, riociguat maintains a relatively normal liver sinusoidal endothelial cells (LSECs) porosity. Subsequent hepatic stellated cell-specific absorption of the anti-fibrosis agent, JQ1 encapsulated in peptide-nanoparticles effectively resolves fibrosis in murine NASH models [199].

### 6.2. Strategies to identify novel NASH targets and their small molecule modulators

Identification and genetic validation of human genetic variants that

**Table 4**
Genetically validated potential NASH therapeutic targets with other novel functions reported since 2022.

| Gene | Full name | Mechanism of action in NASH progression | Reference |
|---|---|---|---|
| ACLY | ATP-citrate lyase | Inhibition of ACLY decreases hepatic steatosis | [178] |
| Aldolase | Aldolase | Inhibition of aldolase activates lysosomal AMPK and ameliorates NASH | [70] |
| 11βHSD1 | 11β-hydroxysteroid dehydrogenase type 1 | Inhibition of 11βHSD1 suppresses liver fibrosis and hepatic steatosis | [179] |
| BNC2 | Basonuclin 2 | BNC2 is a transcription factor that promotes the expression of fibrotic genes and is required for myofibroblastic activation | [180] |
| CD47 -SIRPα | Signal-regulatory protein α | Upregulation of CD47-SIRPα impairs necroptotic hepatocytes by liver macrophages contributes to fibrosis in NASH | [181] |
| DUSP22 | Dual specificity phosphatase 22 | DUSP22 prohibits downstream activation of ERK1/2 and NF-κB cascades to suppress NASH | [182] |
| Dyrk1b | Tyrosine phosphorylation-regulated kinase 1b | Dyrk1b activates mTORC2 to induce steatosis and fibrosis | [117] |
| EphB2 | Ephrin type B receptor 2 | EphB2 induces cell-autonomous inflammation | [183] |
| HSD17B13 | Hydroxysteroid 17-beta dehydrogenase 13 | HSD17B13 protects against liver fibrosis by inhibition of pyrimidine catabolism in NASH | [168] |
| LAPTM5 | Lysosomal-associated protein transmembrane 5 | LAPTM5 interacts with CDC42 and promotes its degradation through lysosome-dependent manner to inhibit MAPK signaling pathway and suppress NASH | [184] |
| MRTF | Myocardin-related transcription factor | MRTF drives fibrosis through integrin-dependent transcriptional reprogramming of myofibroblast cytoskeleton and motility | [161] |
| MSR1 | Macrophage scavenger receptor 1 | MSR1 induces a pro-inflammatory response via the JNK signaling pathway | [185] |
| NTRK3-NT3 | Neurotrophic receptor tyrosine kinase 3-neurotrophin 3 | Pharmacological inhibition of NTRK3-NT3 reverses advanced murine NASH fibrosis | [186] |
| SIRT6 | Histone deacetylase Sirtuin 6 | Downregulation of PSD3 mitigates liver steatosis, inflammation | [187] |
| SRSF1 | Serine-arginine-rich splicing factor 1 | SRSF1 is essential for hepatocyte function and survival by maintaining mRNA transcription and protein synthesis | [188] |
| TREM2 | Triggering receptor expressed on myeloid cells 2 | TREM2 in macrophage facilitates its localization to sites of hepatocellular damage, inflammation and fibrosis, thereby protecting the mice from NASH | [189] |
| UGDH | UDP-glucose 6-dehydrogenase | UGDH suppress RIPK1 kinase-dependent hepatocyte apoptosis during NASH-associated liver damage | [190] |
| VMP1 | Vacuole membrane protein 1 | Hepatocyte-specific deletion of Vmp1 impairs secretion of very low density lipoprotein and stimulates hepatic steatosis | [191] |
| Xbp1 | X-box binding protein-1 | Hepatocyte and macrophage-specific Xbp1 deficiency inhibited the development of steatohepatitis and inflammation | [126] |

tightly associated with NASH progression proved to be effective in identifying new NASH targets. Among the most prominent ones are PNPLA3, HSD17B13, MTARC1, MBOAT7, etc [200]. Other future strategies may need to focus on the root causes that drive NASH progression, especially the transcription factors that reprogram the signaling pathways in hepatocytes and govern the intrahepatic communication, thereby driving the pathogenesis of NASH [41]. Traditionally it is highly challenging to develop small molecule modulators for transcription factors. Nevertheless, a new strategy in cancer research could shed light on targeting transcription factors to treat NASH: Pharmacological disruption of protein-protein interaction at the interface blocks the transcription of target genes. For instance, the inhibitors for transcription factor TEAD have entered phase I clinical trials to treat cancer [201]. Given the fact that YAP/TAZ-TEAD transcription axis drives the fibrosis via activation of Notch pathway, the disruption of the complex in hepatocytes by verteporfin, the inhibitor significantly downregulates the expression of profibrotic gene in NASH progression [202]. The proof-of-concept study is worthy of further investigation to treat NASH.

Furthermore, some pathogenic genes in NASH also promote the progression of other diseases, repurposing of their drugs may be an effective solution, as their dosing, pharmacokinetic properties and adverse effects are well documented.

### *6.3. Human hepatocyte organoids and the application of CRISPR screen to guide the drug discovery of NASH*

Most of the studies on pathogenesis of NASH and therapeutics are predominantly based on animal models, which are not scalable for high-throughput screening of drugs. The interspecies difference between human and rodents also complicated the interpretation of the results. Human liver organoids with self-renewing capability have been established and well-documented to serve as the model for the study of liver function [203]. The organoids were generated from the liver cells in the patients with NASH, who underwent surgeries and they better mimic the NASH pathology in patients, compared to cell lines, and murine models [92]. Compared with patient-derived tissues, liver organoids from human pluripotent stem cells contain hepatocyte-, stellate-, and Kupffer-like cells and they recapitulate the transcriptomic profiles of *in vivo*-derived tissues, and are more facile to establish [204]. In support of this, human NASH liver organoid culture enables the verification of the anti-mitophagy activity of TNFα/Miz1-positive feedback loop [92].

Besides the verification of mechanisms underlying in NASH pathogenesis, the establishment of liver organoids enables setup of the screen to identify drugs to treat HCC, to analyze the molecular feature of drug response and to predict potential drug combination [205]. Liver organoids also facilitate genetic manipulation to alter the metabolic phenotypes *ex vivo*, especially CRISPR-Cas9 screen. Genome-wide CRISPR-Cas9 knockout on L02 cell line successfully identifies CYP46A1 as the key gene that represses lipid droplet accumulation and dysfunctional mitochondrial function during NAFLD progression [206]. Furthermore, FatTracer, the human-derived free fatty acid-induced steatosis liver organoids has been developed as reported in *Nature Biotechnology* [207], when combined with the CRISPR screening platform. Moreover, APOB and MTTP are ablated by CRISPR-Cas9

technology to generate genetic steatosis organoid models. The organoid models were exposed to the drugs and the anti-steatosis potencies of the drugs were assessed based on the drug response, efficacy, transcriptomic profiling and side effects. It thus provides a comprehensive profiling of the drug action in liver. Finally, the CRISPR-Cas9 screen is conducted to identify novel genes that regulate lipid metabolism. Notably, FADS2, the delta-6 desaturase catalyzing the rate-limiting step in the biosynthesis of polyunsaturated fatty acids, is identified as a key mediator to inhibit steatosis. This FatTracer strategy hus serves as the unprecedented platform for identification of genes that regulate novel pathways in NASH and facilitates the future drug discovery.

In summary, the recent multidisciplinary advances in chemical biology, genetics, medicinal chemistry and structural biology significantly accelerate the discovery of drug targets and their small molecule modulators for NASH treatment. The new horizon is beyond the well-established pathogenic pathways. Moreover, the strategies that critically discussed in this article may also shed new insights into the drug discovery of other metabolic disorders to treat obesity, diabetes and NAFLD.

## CRediT authorship contribution statement

**Yibing Wang:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Hanhan Yu:** Writing – original draft. **Zhipeng Cen:** Writing – review & editing. **Yutong Zhu:** Writing – review & editing. **Wenyi Wu:** Writing – review & editing.

## Declaration of competing interests

The authors declare no competing interests.

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