Review

MOLECULAR METABOLISM

Nuclear hormone and peptide hormone therapeutics for NAFLD and NASH

Brian Finan*, **Sebastian D. Parlee**, **Bin Yang**

ABSTRACT

Background: Non-alcoholic steatohepatitis (NASH) is a spectrum of histological liver pathologies ranging from hepatocyte fat accumulation, hepatocellular ballooning, lobular inflammation, and pericellular fibrosis. Based on early investigations, it was discovered that visceral fat accumulation, hepatic insulin resistance, and atherogenic dyslipidemia are pathological triggers for NASH progression. As these pathogenic features are common with obesity, type 2 diabetes (T2D), and atherosclerosis, therapies that target dysregulated core metabolic pathways may hold promise for treating NASH, particularly as first-line treatments.

Scope of Review: In this review, the latest clinical data on nuclear hormone- and peptide hormone-based drug candidates for NASH are reviewed and contextualized, culminating with a discovery research perspective on emerging combinatorial therapeutic approaches that merge nuclear and peptide strategies.

Major Conclusion: Several drug candidates targeting the metabolic complications of NASH have shown promise in early clinical trials, albeit with unique benefits and challenges, but questions remain regarding their translation to larger and longer clinical trials, as well as their utility in a more diseased patient population. Promising polypharmacological approaches can potentially overcome some of these perceived challenges, as has been suggested in preclinical models, but deeper characterizations are required to fully evaluate these opportunities.

Keywords Nuclear hormone; Peptide hormone; NAFLD; NASH; Clinical data

1. PATHOLOGY OF NAFL AND NASH AND HORMONE TREATMENT PARADIGMS

At its basis, the liver is responsible for two essential physiological functions: xenobiotic metabolism and energy metabolism. The contributing role of the liver in energy metabolism and fuel partitioning places it at the pathophysiological precipice, either as cause or causality, of metabolic syndrome. The hepatic manifestation of metabolic syndrome is non-alcoholic fatty liver disease (NAFLD), a continuum of varying and progressive liver pathologies ranging from hepatic steatosis, termed non-alcoholic fatty liver (NAFL), to non-alcoholic steatohepatitis (NASH). Excessive hepatic fat content, a clinical marker of NAFL and NASH, largely originates from an abundance of circulating lipids due to superfluous energy consumption, improper storage and utilization in adipose depots, and subsequent spillover into the circulation. The resulting lipid spillover leads to ectopic deposition in the liver as well as other metabolic organs that are not equipped to adequately store fat, where metabolic derangements such as abnormal nutrient handling and insulin resistance can cause or exacerbate prevailing conditions [1]. Treatment paradigms that specifically target the disposition of fat in adipocytes to address this disrupted energy balance have proven effective in managing NAFL [1]. Although the exact pathogenic drivers of NAFL to NASH progression are heterogeneous, an important trigger is an inability of the liver to handle the excessive influx and subsequent export of lipids. The consequential accumulation of these lipids and an overwhelmed capacity to metabolize them can induce lipotoxicity, oxidative stress, hepatocellular injury, and fibrogenesis that ultimately impede hepatocyte health and NASH progression. NASH is thus histologically characterized by hepatocyte injury and ballooning, inflammation triggered by infiltrated macrophages and Kupffer cells, and pericellular fibrosis induced by the production of collagen by activated stellate cells and myofibroblasts. If uncontrolled, NASH can progress to end-stage liver failure, cirrhosis, and hepatocellular carcinoma, all of which can ultimately prove fatal. NAFL and NASH are both associated with metabolic co-morbidities including obesity, type 2 diabetes (T2D), dyslipidemia, and hypertension and are associated with an increased risk of cardiovascular disease and morbidity. Likewise, environmental cues influence disease progression and genetic risk factors such as PNPLA3 are associated with the full spectrum of histological NAFLD pathogenesis [2].

Despite substantial progress in understanding the molecular etiology and progressive clinical pathology of NASH, significant challenges in the discovery of therapeutic targets remain as no drug is currently approved worldwide that is indicated for NASH. These challenges are diverse, numerous, and can impact the entire therapeutic discovery process and evaluation. At the most basic level, optimized *in vitro* [3] and *in vivo* [4] models that recapitulate the human condition are progressing, but their utility to identify novel targets and predict translational efficacy are still lacking. This is at least in part due to the

Novo Nordisk Research Center Indianapolis, Inc., United States

*Corresponding author. Novo Nordisk Research Center Indianapolis, Inc., Indianapolis, IN, 46241, United States. E-mail: BFIN@novonordisk.com (B. Finan).

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heterogeneity of the clinical disease presentation and lack of concrete clinical comparisons. Clinical trials have their own set of unique challenges that include imprecise and often biased methods to diagnose NASH and assess its regression [5]. Liver biopsy and histopathological assessment are common methods of diagnosing and evaluating therapeutic intervention, but even pragmatic considerations regarding the inherently variable method of how histopathological samples are adjudicated, analyzed, and interpreted before and after therapy across multi-center studies can influence the judgment of clinical performance [6]. While standardization of specimen processing and centralized reading processes are being explored, machine learning is being leveraged as a straightforward opportunity to accelerate a more comprehensive yet unbiased histological assessment [7,8]. In contrast, non-invasive biomarkers such as serum alanine transaminase (ALT), collagen fragment N-terminal type III collagen pro-peptide (PIIINP or PRO-C3), and enhanced liver fibrosis (ELF) tests, a composite of hyaluronic acid, PIIINP, and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), have been used to estimate therapeutic responses in clinical trials of NAFL and NASH. Heterogeneity in clinical manifestations of this spectrum disorder [9] present challenges in defining these as precise biomarkers that can be used as surrogates for improved liver and patient health. Extensive research continues to identify new predictive and non-invasive serological [10–12] biomarkers of NASH risk, status, and response to therapy. Despite the challenges and the need for standardized methodologies and technical development of supporting methods, advancement of non-invasive techniques as an alternative to invasive histology, which include tunable biosensors to estimate tissue damage, magnetic resonance imaging of proton density fat fraction (MRI-PDFF) to quantify hepatic fat content [13], and elastography to estimate liver stiffness [14], continue to be evaluated in the clinic and are catalyzing additional research. Overall, the sequential combination of numerous non-invasive tests has the potential to improve specificity and selectivity to discriminate disease status at baseline and provide more granularity to understand responses to therapy [15].

In addition to challenges with diagnostic measurements, substantial variability in clinical trial designs with respect to patient inclusion criteria, including baseline disease status of obesity, dyslipidemia, type 2 diabetes (T2D), composite NAFLD activity scores (NAS), and fibrosis stage (stages F1–F4) also complicate the comparative clinical evaluation of different therapeutic modalities. Furthermore, primary and secondary readouts, notably whether NASH resolution or fibrosis improvement should be the primary outcome in clinical trials, differ across studies and could benefit from uniformity for regulatory considerations and cross-trial comparisons. Classical, regimented clinical trial designs may therefore need to be revisited to be more adaptive. For regulatory approval of new drugs for NASH, investigational therapy must either show: (i) resolution of NASH (0 score for hepatocellular ballooning and 0–1 score for lobular inflammation) without worsening of fibrosis or (ii) a one-stage improvement in fibrosis without worsening of NASH. Clinical trials can therefore be radically different in design to achieve either endpoint, irrespective of the limitations inherent in the assessment of these clinical endpoints. Despite value in clinical trial uniformity, it can present inherent challenges as the specific therapy mechanisms must be considered in trial designs and evaluations. For instance, therapies targeting the metabolic derangements in NASH may not show improved fibrosis in short-term clinical trials since fibrosis reversal is a lengthy process that likely requires long-term clinical trials to prove effective. In this case, NASH resolution as opposed to fibrosis improvement is the optimum primary readout for this class of therapeutics despite fibrosis being an important predictor of mortality in NAFLD [16]. To this end, NASH resolution seems to correlate with an increased probability of fibrosis improvement as observed across select trials studying metabolically targeted therapeutics [17], although more retrospective analyses from emerging clinical trials are required to solidify these observations.

Lifestyle modification is a necessary and standard of care for all NAFL and NASH patients irrespective of the disease state or therapeutic intervention. As there are no therapies worldwide that are specifically approved to treat NASH, treatment of comorbidities is the foundation for current medical options. Even the medical care approach can be complicated as it is often integrated between hepatologists for advanced liver diseases and endocrinologists to address the underlying metabolic syndrome. However, the cornerstone for care is largely weight loss as it is established that a 5% reduction in body weight is associated with a 26% relative reduction in liver fat, improved necro-inflammatory injury, and higher propensity for NASH resolution [18,19]. Intensive lifestyle modification resulting in $>$7% body weight loss improves liver histology in biopsy-proven NASH patients [20], and the percent weight reduction induced by lifestyle modification correlates with improved NASH histologic parameters [19]. Importantly, of patients who lost $>$10% body weight, 90% demonstrated resolution of NASH and nearly half showed a regression of fibrosis. The greatest challenge with lifestyle modification is that a relatively small proportion of individuals can achieve sustained weight loss greater than 10% [21]. While a systematic meta-analysis of randomized clinical studies showed a consistent association of weight loss to improve hepatic steatosis and transaminase levels [22], weight loss interventions were not associated with reduced fibrosis and there is limited evidence of long-term liver health benefits. Given the difficulty of sustained weight loss via lifestyle modification alone, several pharmacotherapies have been developed and are now approved for weight loss. Of the currently approved anti-obesity drugs, the efficacy of placebo-corrected body weight loss ranges from 5 to 10% [23], although promising clinical data are continuing to emerge suggesting $>$10% sustained body weight loss can be achieved with next-generation therapies [24,25]. The efficacy and long-term prognosis of bariatric surgeries remain higher relative to medical treatment [26,27], but direct comparisons have yet to be made to these emerging therapeutic options. Regardless, weight loss associated with bariatric surgeries can be viewed as a primary driver for metabolic improvements, notably improved insulin resistance [28]. Thus, as a therapy, bariatric surgery has the potential to address key pathogenic nodes across NAFLD. In line with this, bariatric surgery resolved NASH without worsening of fibrosis in 84% of patients with biopsy-confirmed NASH, and a gradual progressive reduction in fibrosis was observed over a 5-year follow-up study [29]. Importantly, NASH resolution occurred early after surgery and was sustained for 5 years, with early surgery success indicating long-term NASH resolution. Despite the substantial upside of bariatric surgeries for resolving NASH, the risk of these surgeries currently precludes their use as first-line therapy for NAFL and NASH. Thus, the requirement for pharmacotherapy options is imperative for first-line therapy in the broader patient population.

To date overall, first-generation drug candidates for NASH have shown relatively modest efficacy in late-stage clinical evaluation, particularly in patients with advanced states of fibrosis. This highlights the challenges of translating short duration phase 2 studies of target engagement and proof of concept in small patient populations into longer, larger, and more diverse phase 3 trials. Despite these limitations, the pipeline of therapies being evaluated in the clinic are numerous and have diverse molecular action. Disruptive innovation is also being explored in NAFL and NASH as a result of the recognized

paucity in therapeutic options. These include but are not limited to gene therapy, stem cell therapy, and transplantation alternatives. Classical therapeutic targets (that is, those targets in which a chemical substance is the active pharmaceutical ingredient) for NAFL and NASH comprise the largest class of therapeutics, and can be classified based on the targeted pathogenic processes: metabolism, inflammation, or fibrosis. Hormonal-based strategies that target the metabolic pathologies of NAFL and NASH have shown the most promise in clinical studies to date, likely because these agents address the root cause of the disease: metabolic overload. The therapeutic strategy of targeting the metabolic pathophysiology of NAFL and NASH is to mediate the disposal of accumulated lipids or lessen lipid substrate delivery to the liver. These hormonal-based strategies mostly include small molecules that target nuclear hormone receptors and large molecules (peptides and proteins) that target cell surface receptors. These hormones have multi-factorial benefits on various aspects of metabolic syndrome, but most have shown notable efficacy to decrease hepatic fat deposition, lower circulating atherogenic lipid species, or decrease excessive body weight. These hormonal-based strategies are advantageous because many have liver-centric mechanisms and extra-hepatic or systemic actions that improve various aspects of metabolic syndrome, thus providing ancillary mechanisms to treat the underlying metabolic milieu of NAFLD (Figure 1).

2. NUCLEAR HORMONE RECEPTORS

Nuclear receptors are generally ligand-dependent transcription factors that regulate numerous core physiological processes. Falling into 7 subfamilies, many of the 48 known nuclear receptors modulate discrete components of the gastrointestinal—hepatic—adipose axis to regulate systemic energy metabolism [30]. The liver is one of the organs responsible for energy substrate metabolism, particularly the regulation of the anabolic and catabolic machinery responsible for cholesterol, lipid, and lipoprotein metabolism. These processes are often tightly regulated by transcriptional pathways under control of nuclear hormones and their receptors. Nuclear hormone analogs that have shown the most promising therapeutic benefit for NASH include thyroid hormones, farnesoid X receptor (FXR) agonists, and peroxisome proliferator-activated receptor (PPAR) agonists. The downstream signaling networks of these nuclear hormones that are involved in modulating cellular pathways relevant to NAFL and NASH pathology were recently reviewed elsewhere [31]. Glucocorticoids, despite their anti-inflammatory properties, have largely not been explored in metabolic diseases such as NASH since glucose production is an undesirable primary effect of engaging this system. Whether glucocorticoid receptor agonists can be rationally designed to harness their anti-inflammatory properties without glucoregulatory properties remains to be extensively studied. Mineralocorticoid receptor antagonists are being pursued for diabetic nephropathy with promising clinical benefits [32]. Although the mechanism of action suggests benefits in NASH, there has been little developmental activity in this space. Mimics of the sex hormones estrogen and testosterone are also being evaluated for their effects on various liver diseases, but the clinical research is early. In this article, the most recent clinical results of thyromimetics, FXR agonists, and PPAR agonists are reviewed and contextualized.

2.1. Thyroid hormone receptor agonists

Produced in the follicular cells of the thyroid gland, thyroid hormones thyroxine (T_4) and triiodothyronine (T_3) are pivotal regulators of diverse physiological processes, including energy metabolism and are strictly regulated by a negative feedback mechanism involving a hypothalamus-pituitary-thyroid (HPT) axis. The predominant circulating thyroid hormone, T_4, is converted to the less stable yet biologically more active form T_3 by regioselective deiodination via iodothyronine deiodinases. Thyroid hormone actions are mediated by two nuclear hormone receptor isoforms, thyroid hormone receptor α (THR-α) and β (THR-β). While both isoforms are expressed in the brain and involved in crucial developmental functions, THR-α is predominately expressed in the heart and skeletal muscle and its activation influences various cardiovascular functions. In contrast, THR-β is predominately expressed in the liver, particularly hepatocytes, where it plays an important role in regulating lipid, cholesterol, and lipoprotein metabolism through multiple integrated pathways.

The importance of thyroid hormones in human metabolism is evident in cases of abnormally heightened or diminished circulating concentrations. Hypothyroidism is associated with decreased thermogenesis and higher body mass, and conversely, hyperthyroidism is associated with lower body weight and lower cholesterol levels [33]. Furthermore, administration of native thyroid hormones (T_3 and T_4) lowers body weight and cholesterol in humans [34]. Elevated thyroid hormone levels are however associated with cardiac abnormalities, notably tachycardia and heart failure [35], presumably through actions at THR-α. Consequently, native thyroid hormones are precluded from their chronic use in humans to treat metabolic conditions as these patients are predisposed to increased risks of cardiovascular disease. Early medicinal chemistry efforts focused on thyromimetics of enhanced selectivity toward THR-β to improve the therapeutic index of native T_3/T_4 with respect to maximize cholesterol-lowering efficacy and minimize cardiovascular effects. A particular challenge therein is the inherent similarity of the two thyroid hormone receptor isoforms, which only differ by a single residue in the active site [36] and the lack of any selective preference of native T_3. Despite these challenges, two first-generation THR-β-selective thyromimetics, including sobetirome (GC-1) [37] and eprotirome ([38]), showed promising efficacy to reduce atherogenic lipids and hepatic steatosis in numerous preclinical models with minimal thyrotoxicity on the HPT axis [39]. The mechanisms of action to decrease liver fat appear pleiotropic and likely a combination of increased β oxidation and reversing mitochondrial dysfunction, both of which have been established as contributing pathogenic processes in chronic liver diseases [40]. Despite these positive effects on hepatic health, clinical development was halted due to adverse effects on bone cartilage observed in dogs following chronic treatment with eprotirome [41]. These adverse effects on cartilage have not been observed in humans nor has the precise mechanism for the effects in dogs been elucidated. Consequently, two next-generation liver-selective thyromimetics (resmetirom and VK2809) with greater THR-β selectivity and liver biodistribution emerged. These analogs have advanced to clinical evaluation where improvements in lipid serology and liver fat content have been consistently demonstrated in NAFLD patients.

Resmetirom, also known as MGL-3196, is a liver-selective and THR-β-selective agonist. Unlike predecessor THR-β ligands, resmetirom is based on a pyridazinone structure, which supports greater selectivity and permits oral bioavailability [42]. The selectivity for THR-β vs THR-α is estimated to be at least 28-fold in favor of THR-β based on *in vitro* coactivator recruitment assays. Of note, resmetirom is decreased in potency by 14-fold at THR-β relative to native T_3. Liver selectivity is governed by a few different factors. First, resmetirom is administered orally, thus first-pass metabolism permits distribution to the liver. Resmetirom also has a high affinity for circulating proteins including albumin, which is naturally cleared through the liver, so a second route

of metabolism supports liver delivery. Third, resmetirom has relatively low extra-hepatic tissue penetration, with an estimated 8:1 liver-to-plasma partitioning ratio. Finally, the high selectivity for THR-β supports semi-selective function in the liver. Holistically, these attributes of resmetirom provide favorable systemic exposure and tissue bias necessary for this class of agents to have an improved therapeutic index, notably with minimal side effects attributed to direct action on the cardiovascular and skeletal musculature system.

In early clinical evaluations of multiple ascending doses in patients with mild elevations of LDL cholesterol, treatment with resmetirom at daily

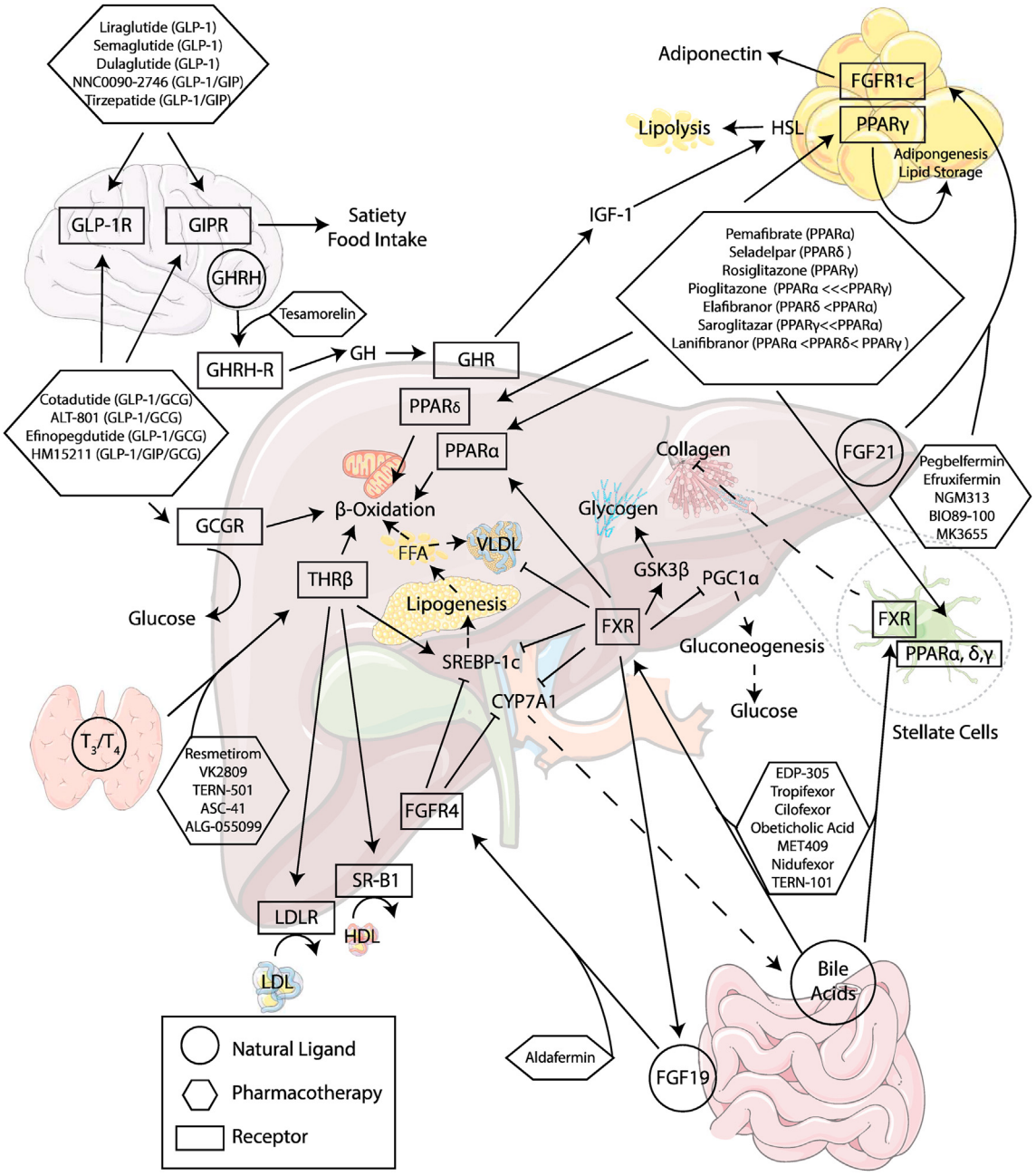

Figure 1: A schematic representation of major regulatory pathways associated with NAFL and NASH clinical candidates. Therapies largely target three organ systems to improve liver health: 1) liver, 2) adipose tissue, and 3) brain. Nuclear hormone-based agonists for THR-β, PPARδ, PPARα, and FXR and peptide-based agonists for GcgR and FGFR4 directly target the liver. Thyroid hormone agonists specifically decrease ectopic fat by binding to hepatic THR-β, leading to mitochondrial β-oxidation and modulation of cholesterol metabolism. A similar pathway is utilized by agonists of PPARδ, PPARα, and glucagon. FXR agonists activate and inhibit diverse signaling pathways that alter glycogen storage, β-oxidation, and circulating FGF19 while simultaneously decreasing lipogenesis, bile acid production, gluconeogenesis, and VLDL-production. FGF19-based therapies mimic aspects of FXR therapy decreasing lipogenesis and bile acid production. Both pan-PPAR and FXR agonist signaling in hepatic stellate cells decrease collagen production to improve fibrosis. Extrahepatic adipose tissue acts as a second target for therapeutic improvement of NAFL and NASH. PPARγ agonists and FGFG21-based therapies signal adipocytes to improve lipid storage and decrease insulin resistance and inflammation. GHRH, GLP-1, and GIP agonists signal the CNS to resolve hepatic dysfunction. GHRH increases GH secretion and subsequent IGF-1 production, which can directly work on adipocytes and hepatocytes to engage multiple metabolic pathways. Incretin-based therapies targeting GLP-1R and GIPR largely improve liver health by decreasing body weight and fat mass via food intake modulation but could also involve secondary effects in adipose tissues via indirect or direct actions.

doses of ≥50 mg for two weeks reduced levels of atherogenic lipid species (triglycerides and LDL cholesterol) [43]. The maximum efficacy observed included a 60% reduction in circulating triglycerides relative to placebo at a dose of 80 mg and a 30% reduction in LDL cholesterol relative to placebo at a dose of 200 mg [43]. Resmetirom was well tolerated without dose-related adverse events with respect to liver enzymes or cardiovascular function. At the highest dose tested, a significant reduction in free T_4 of 10–20% was observed without changes in other serological markers of thyroid function, suggesting the lower T_4 level was not due to direct engagement of the negative feedback loop of the HPT axis. These effects on T_4 were only evident at doses (100–200 mg) above the maximally efficacious dose (80 mg) in this 2-week study, and the magnitude of this effect did not suggest resultant clinical ramifications. A similar phenomenon was also noted in preclinical testing in rodent models. One plausible mechanism for this reduction in circulating T_4 levels is direct negative feedback hepatic action via THR-β to increase deiodinase 1 levels or function. Free T_3 levels were however unchanged, thus countermanding this possibility. Increased hepatic or renal uptake of free T_4 could also contribute to this effect, as could a speculative and undefined hepatic feedback mechanism on HPT function.

Resmetirom was studied in a 36-week phase 2 clinical trial in biopsy-confirmed NASH patients who were treated with daily oral doses up to 80 mg [44]. Resmetirom caused a significant reduction from baseline in relative and absolute hepatic fat fraction in these NASH patients throughout the course of treatment. Placebo-corrected relative hepatic fat content was reduced by 28.8% after 36 weeks across all of the doses tested, and a greater proportion of patients on resmetirom therapy achieved ≥30% reduction in liver fat relative to placebo (67.6% vs 29.4%). A higher proportion of patients achieved NASH resolution with resmetirom therapy than placebo controls (24.7% vs 6.5%). In line with earlier studies, atherogenic lipids such as LDL cholesterol (−17.3% from placebo at the end of the study) and triglycerides (−36.0% from placebo at the end of the study) were reduced from baseline with resmetirom treatment. Serum levels of ALT and AST were also significantly reduced, as were markers of fibrosis and fibrogenesis such as PRO-C3. Resmetirom did not influence body weight in these overweight patients, although weight loss appeared to enhance resmetirom's response to decrease hepatic fat fractions based on a post hoc analysis. It was however concluded that the beneficial effects on liver fat and steatohepatitis were not driven by weight loss. Unlike what was demonstrated with predecessor thyromimetics, no significant adverse effects on levels of thyroid-stimulating hormone, bone mineral density, heart rate, or biomarkers of diabetes or cardiovascular performance were observed with resmetirom therapy. Resmetirom (80 and 100 mg) is currently being investigated in a phase 3 trial in NAFLD patients characterized by non-invasive imaging and biomarkers (MAESTRO-NAFLD1; NCT04197479) and in NASH patients with fibrosis stages 2–3 (MAESTRO-NASH; NCT03900429).

VK2809 (formerly known as MB07811) is a liver-selective THR-β prodrug agonist with a phosphonate-containing thyromimetic as the active metabolite (formerly known as MB07344). The binding affinity (Ki) of the prodrug VK2809 is 14.6 μM at THR-β and 12.5 μM at THR-α, whereas the active drug/metabolite has a binding affinity of 3.0 nM at THR-β and 35.2 nM at THR-α, thus rendering it nearly 12-fold selective for THR-β vs THR-α [45]. Of note, the potency of the active phosphonate thyromimetic decreases by ∼10-fold relative to native T_3 at THR-β. The phosphonic acid moiety is also thought to convey more liver preference relative to systemic distribution by nature of the charged state when at neutral pH, which enhances active cellular transport by organic anion transporters that are enriched in hepatocytes. The HepDirect prodrug [46] employed on this semi-selective THRβ agonist provides additional liver selectivity. The HepDirect prodrug is based on a substituted cyclic phosphonate diester that is cleaved by cytochrome P450 yet is highly stable in circulation. Thus, the prodrug is converted into an active drug once in the liver and only in hepatocytes due to the selective presence of cytochrome P450. The traceless mechanism for prodrug decomposition is a two-step process initiated by CYP3A4-mediated oxidative ring opening to form a linear monoacid that is subsequently converted into the phosphonate-bearing active drug [46]. Importantly, the intermediates and byproducts of this reaction are retained within hepatocytes by nature of the charge states, which limit systemic adverse side effects of the byproducts. An ancillary benefit of this specific HepDirect prodrug is improved oral bioavailability as the phosphonic acid group seemingly impairs gastrointestinal permeability. VK2809 was shown to undergo first-pass hepatic metabolism and hepatic prodrug conversion to release a negatively charged thyromimetic, which itself has poor distribution to tissues and ultimately is eliminated in the bile. Overall, the estimated therapeutic index for VK2809 is >125 for cholesterol lowering relative to cardiovascular effects but only ∼7.5 with respect to negative feedback on the HPT axis [45].

Similar to early clinical results with resmetirom, in a phase 1 clinical trial of VK2809, dose-dependent reductions in LDL cholesterol and triglycerides were observed up to a dose of 20 mg. In a small phase 2a trial (NCT02927184) in patients with NAFLD and hypercholesterolemia, interim results suggested that 10 mg of VK2809 resulted in a significant reduction from baseline in LDL cholesterol (>20% placebo-adjusted) after 12 weeks of treatment [47]. Other atherogenic lipids, notably apolipoprotein B (ApoB), were likewise reduced relative to placebo. The combined dose levels of VK2809 reduced placebo-corrected relative liver fat by 47.1%, and a greater proportion of patients on VK2809 therapy achieved a ≥30% reduction in liver fat relative to placebo (87.9% for VK2809 and 16.7% for placebo). Heart rate, blood pressure, cardiovascular safety biomarkers, body weight, and serological markers of the HPT axis were not affected by VK2809 treatment. The robust efficacy observed in this trial, which included liver fat content and reductions in atherogenic lipids irrespective of baseline characteristics and risk factors, suggested potential protection from cardiovascular risks in these NASH patients. A phase 2b study of VK2809 at 4 dose regimens (daily doses of 1.0 mg or 2.5 mg and every other day doses of 5.0 mg and 10.0 mg) in patients with biopsy-confirmed NASH and fibrosis stages 1–3 is presently underway (VOYAGE; NCT04173065).

Of the current nuclear hormone agonists in clinical evaluation, THR-β agonists seemingly have the greatest efficacy to lower hepatic fat content in NAFL patients. Furthermore, the effect sizes to lower atherogenic lipids suggest a potential upside in decreasing the risk of CVD progression in NAFL patients. A next wave of thyromimetics are entering into clinical studies, including ASC-41, ALG-055099, and TERN-501, which may have improved selectivity and pharmacokinetic profiles. As thyromimetics essentially work by removing the underlying etiological cause of hepatocyte injury (hepatic lipids and hepatic insulin resistance) by direct hepatocyte action, current knowledge suggests a minimal impact to reverse fibrosis as suggested by the lack of observed histological improvement in fibrosis following treatment with resmetirom. This may result from a mechanism of action restricted to hepatocytes, but it cannot be concluded whether this is the result of inadequate statistical power, trial length, or low-grade fibrosis at baseline in enrolled patients. Ultimately, there may be limited utility in using thyromimetics in patients with later-stage fibrosis and NASH,

thus patient segmentation to only NAFL patients with low-grade fibrosis may be necessary. Combination with other agents that selectively target inflammatory and/or fibrotic mechanisms may also provide an opportunity to address various stages of disease. Despite a relatively inert safety profile for resmetirom, small changes in serum thyroxine were observed, so it will be important to see if this manifests in longer-term trials to understand whether patients at risk for cardiac arrythmias or osteoporosis should be precluded from use.

2.2. FXR agonists

FXR is a pleiotropic nuclear hormone receptor for bile acids that is enriched in the liver, kidney, and intestines. By heterodimerizing with retinoid X receptor (RXR) and binding FXR response elements in promoters of the target genes, FXR directly governs a number of enterohepatic metabolic processes including biosynthesis of bile acids, cholesterol, and triglycerides [48]. For instance, activation of FXR decreases transcription of *CYP7A1*, leading to decreases in 7-α-hydroxy-4-cholesten-3-one (C4) and bile acids. Similarly, FXR agonism decreases transcription of sterol regulatory element-binding protein 1c (*SREBP1c*), which directly and indirectly reduces synthesis of various lipid precursors and species such as unsaturated fatty acids. Activation of hepatic FXR by lipophilic bile acids can also modulate glucose production to improve glucose homeostasis and insulin sensitivity by altering genes including *PGC1*α and *GSK3*β [49,50] as well as modulating glucagon reciprocal responses [51]. Activation of intestinal FXR also promotes the secretion of fibroblast growth factor 19 (FGF19), further modulating aspects of cholesterol metabolism, bile acid synthesis, and lipid oxidation via hepatic FGF receptor 4 (FGFR4) [52]. In addition to improving metabolite handling, FXR expression is also detected in quiescent and activated hepatic stellate cells and is associated with decreased collagen production, supporting a role in fibrosis regression [53]. Despite clear directions in which way to modulate hepatic FXR action for beneficial pharmacological effects, debate exists about whether to agonize or antagonize intestinal FXR for systemic metabolic benefits [54,55]. Nevertheless, bile acid modulation, predominantly via FXR agonism, is the modality with the greatest number of drug candidates currently being explored for NASH. Strategies employed to improve the pharmaceutical properties of FXR agonists mostly focus on maintaining high potency while trying to mitigate adverse effects on pruritus, LDL cholesterol, and liver toxicity. This is largely achieved by altering the chemical structure to influence FXR engagement and tissue distribution as well as exploring non-bile acid-derived structures.

Bile acids do not signal through FXR alone; they can also signal through a membranous G protein-coupled receptor (Takeda G protein-coupled receptor 5; TGR5), which is reported to be expressed in Kupffer cells and hepatic endothelial cells but not hepatocytes [53]. Based on this expression pattern, activation of this receptor is believed to modulate inflammatory processes. TGR5 is also expressed in other metabolically active tissues, including brown adipocytes, where pharmacological activation has improved systemic metabolism and body weight in rodents [56,57]. TGR5 activation in these extra-hepatic tissues may positively impact hepatic steatosis via indirect actions. TGR5 activation has also been linked to pruritus observed following treatment with bile acid derivatives [58]. Decoupling the positive metabolic actions and secondary effects of FXR vs TGR5 agonism requires more intensive study, particularly via clinical evaluation of selective ligands that are emerging from discovery research [59].

Obeticholic acid (6-ethylchenodeoxycholic acid; INT-747) is a semi-synthetic analog of chenodeoxycholic acid and a selective and potent agonist for FXR. As passive diffusion through cell membranes is important for activation of FXR, both cell-free and cellular assays are often utilized to characterize FXR agonists. Obeticholic acid has similar potency in functional assays with a reported EC_{50} $\sim$90 nM, which is nearly 100-fold more potent than its natural bile acid precursor [60]. In a phase 1 study that investigated 25 mg and 50 mg of obeticholic acid for 6 weeks in patients with T2D and NAFLD [61], insulin sensitivity improved by 24.5% across both doses compared to a 5.5% worsening in the placebo group. However, obeticholic acid treatment increased circulating levels of LDL cholesterol, which was likely driven by increased FGF19 production and/or downregulation of *CYP7A1*. In a phase 2 trial investigating obeticholic acid (25 mg) for 72 weeks in patients with non-cirrhotic NASH (FLINT; NCT01265498) [62], 45% of patients who received obeticholic acid showed improved liver histology from baseline biopsy compared to 23% from placebo as determined by a $\geq$2 decrease in NAS without worsening of fibrosis. All individual components of NASH histology, notably steatosis, hepatocellular ballooning, and inflammation improved. While an improvement in fibrosis was evident ($\sim$11% placebo-adjusted), the effect size remained small. Serum levels of ALT and γ-glutamyl transferase (GGT) decreased with obeticholic acid treatment relative to placebo. Despite these improvements in histological features of NASH including steatosis, hepatocellular ballooning, lobular inflammation, and fibrosis, the number of patients who achieved NASH resolution failed to significantly differ from placebo. In addition to these measurements of liver health, obeticholic acid induced a body weight loss of $-$2.2 kg ($-$2.3%) relative to placebo along with a small decrease in blood pressure. Unlike in the shorter phase 1 trial, obeticholic acid was found to increase fasting insulin levels and worsen hepatic insulin resistance (measured by HOMA-IR) compared to placebo. Obeticholic acid treatment was also associated with increased concentrations of LDL cholesterol, decreased HDL cholesterol, and no effect on serum triglycerides in this trial [62].

Within the REGENERATE trial (NCT02548351) in patients with NASH and fibrosis stages 1$-$3 [63], interim readouts showed that obeticholic acid (25 mg) improved fibrosis by at least one stage without worsening of NASH in 23.1% of patients relative to 11.9% of patients who received placebo. Similar to the previous study, resolution of NASH was achieved in 12% of patients on obeticholic acid, which was not statistically significant to the 8% resolution observed with placebo. Of note, the REVERSE trial studying obeticholic acid in fibrosis stage 4 NASH patients with compensatory cirrhosis is underway. Obeticholic acid therapy was again associated with as much as a 25% increase in LDL cholesterol from baseline. This increase in LDL cholesterol is a significant concern for the utility of obeticholic acid, particularly since NASH patients are already at a higher risk of cardiovascular disease. Treatment with obeticholic acid was accompanied by higher rates of pruritus, which is a preexisting symptom associated with cholestatic disorders such as NASH. Further exacerbation of this side effect may limit the use of obeticholic acid in susceptible patients. Whether these side effects are compound-specific (bile acid structure), mechanism-specific (FXR activation vs TGR5 activation), or tissue-specific (dermis vs hepatic) remains to be studied, but comparisons across trials of novel FXR ligands may provide insights.

EDP-305 is a potent steroidal FXR agonist developed with the ambition of fewer adverse side effects on pruritus and hepatic injury by nature of its structure that prevents the formation of conjugated metabolites [64]. Interim results from a phase 2 trial in patients with NASH demonstrated a placebo-corrected decrease in relative hepatic fat content of 18.6% and a decrease in levels of ALT with the highest dose tested (2.5 mg). Pruritus was however reported in 51% of patients who received the effective dose of 2.5 mg. LDL cholesterol only showed an

increasing trend, but HDL cholesterol decreased at the 2.5 mg dose [65]. These findings suggested that the severe itching side effect of these bile acid analogs is not due to drug metabolism events but rather specific to the mechanism of action.

Tropifexor is a highly potent (EC_{50} ~200 pM in cell and cell-free assays) non-bile acid FXR agonist [66]. In healthy obese patients, tropifexor (10–100 μg daily) dose-dependently increased FGF19 with a concomitant decrease in levels of C4, markers for FXR engagement, but did not reduce serum triglycerides over 14 days of therapy [67]. Unlike the increased prevalence of pruritis observed with obeticholic acid therapy, tropifexor was not associated with pruritis in this short trial in healthy patients, which may be a result of the short trial length. Interim results from an ongoing phase 2 clinical trial in NASH (FLIGHT-FXR; NCT02855164) demonstrated decreases in relative hepatic fat fractions (31% and 39% at each dose level vs 3% for placebo) and serum levels of ALT at higher doses (140 and 200 μg) after 48 weeks of treatment [68]. However, a persistent increase in LDL cholesterol at all dose levels relative to placebo was observed, and an additional interim analysis suggested a failure to meet histological endpoints on NASH resolution and fibrosis improvement. Although the lower trends of pruritis require confirmation in longer clinical trials and despite the unknown mechanism of the induced pruritus, the lack of TGR5 engagement and non-bile acid structure of tropifexor lend support that the lack of pruritis will persist with more rigorous clinical testing.

One hypothesis is that partial FXR agonists could differentiate from full FXR agonists regarding efficacy and safety. A recently reported partial FXR agonist (nidufexor), which is approximately 10-fold less potent with 50% partial FXR agonism relative to tropifexor [69], is currently in clinical evaluation in patients with NASH (NCT02913105) and diabetic nephropathy (NCT03804879). Preliminary results showed decreased levels of ALT up to 25% relative to placebo and reduced hepatic fat by 32% (placebo-corrected) at a 100 mg dose [70]. However, the number of pruritus events (54.1%) was still evident and HDL cholesterol decreased by up to 16% despite no change in triglycerides or LDL cholesterol. These data suggest that the proposed mechanism of altering the dynamics of the interaction with FXR to promote transcriptional partial agonism does not provide an alternative path forward to improve the therapeutic index of FXR agonism.

Cilofexor is a potent (EC_{50} ~15 nM in cell-free assays; ~40 nM in cell assays) non-steroidal FXR-selective agonist that seemingly does not enter the enterohepatic circulation, thus strictly acting through intestinal FXR to induce FGF19 secretion [71]. The hypothesis is that selective activation of intestinal FXR will mitigate potential liabilities such as pruritus and hepatic toxicity. In a phase 2 trial in NASH patients [71], 24 weeks of treatment with cilofexor (100 mg) reduced relative liver fat by 24.6% relative to placebo and 39% of patients achieved a ≥30% reduction in relative liver fat content vs 13% of patients on placebo. However, liver stiffness and serological changes in biomarkers of fibrosis (ELF scores) were not changed by cilofexor therapy despite decreased levels of GGT and C4. This suggests that direct hepatic signaling is likely necessary for NASH resolution. Of note, pruritis was still evident in patients on the highest dose of cilofexor, suggesting either a dose-dependent effect to escape the gastrointestinal tract or that the secondary mechanism of intestinal FXR activation governs pruritus. By nature of intestinal FXR activation to induce FGF19 secretion, the likelihood of increasing LDL cholesterol persists if the resultant exposure levels of FGF19 are above the threshold. If increases in LDL cholesterol can be mitigated based on this pharmacological profile, there is a considerable upside for this intestinal FXR mechanism as part of a combination therapy.

MET409 is non-bile acid structure FXR agonist with sustained action. After 12 weeks of treatment in biopsy-confirmed NASH patients, MET409 lowered relative hepatic fat content by 49% and 32% relative to placebo at 80 and 50 mg doses, respectively [72]. Pruritus was reported in 35% (80 mg) and 10% (50 mg) of patients, and LDL cholesterol again increased by 23.7% (80 mg) and 7.8% (50 mg). Although the data suggests an improved therapeutic index can be achieved with chemical structure optimization, a seemingly narrow therapeutic window still exists, particularly with respect to the atherogenic lipoprotein profile that may limit its medicinal utility in these patients.

As previously mentioned, the clinical efficacy of FXR agonists in more advanced NASH patients is relatively modest albeit measurable. This suggests that FXR agonists may have limited utility as a standalone therapy, but seemingly an important ingredient of an effective combination therapy. Although pruritus observed with most FXR agonists is evident, it can be considered more of a patient inconvenience rather than an adverse side effect. The prevailing limitation of FXR agonists is the potential to increase atherogenic lipids, notably LDL cholesterol. As NAFLD increases the risk of CVD [73], a decrease in atherogenic lipids is desirable for pharmacotherapy and an increase brought on by treatment is certainly unacceptable. There is potential to adjunctively address increased LDL cholesterol with statins or other cholesterol-lowering agents, but this approach can present practical challenges such as drug–drug interactions that have yet to be extensively studied. Akin to medicinal chemistry approaches leveraged for THR-β-selective thyromimetics, selective FXR modulators, including gene-selective and tissue-selective targeting, have promise but are yet to be proven to dissociate the beneficial metabolic mechanisms from unwanted inflammatory and atherogenic liabilities. Liver-selective targeting of FXR ligands seems conceptually achievable since chemical appendages on thyromimetics have afforded preferential liver delivery (as previously described), although subtle modifications to the prodrug structures would be required. However, the practicality of this approach depends on the notion that the increase in atherogenic lipids is the result of extra-hepatic activation of FXR, which has yet to be conclusively proven in humans. More challenging from a drug design perspective, but with more potential upside, is engineering FXR ligands with biased activity toward specific gene regulation by targeting (i) differential cofactor interactions, (ii) differential binding to alternative DNA motifs, (iii) differential transactivation/transrepression mechanisms, and (iv) differential ligand-binding sites for allosteric modulation. To aid in the pursuit of these selective FXR ligands, a deeper understanding is required of the structure–activity relationship of ligand-FXR interactions in diverse biological assays. Lessons learned from the mechanisms of glucocorticoid receptor dimerization [74], cofactor interaction dynamics [75], and selective activation [76] that decouple transcriptional cascades can serve as an inspiration.

2.3. PPAR agonists

PPARs are a superfamily of nuclear hormone receptors that are activated by various lipid species including free fatty acids, eicosanoids, and prostaglandins. The three subtypes include PPARα, PPARδ, and PPARγ, each with pleiotropic functions that include the regulation of multiple lipid metabolism pathways. PPARα is highly expressed in metabolically active tissues including the liver where it regulates fatty acid uptake, lipid oxidation, and triglyceride turnover. In contrast, PPARδ is more selectively expressed in muscle and immune cells and in lower levels in the liver where it modulates mitochondrial function and fatty acid oxidation. PPARγ is predominantly expressed in adipocytes but has ubiquitous low expression throughout various tissues.

In adipocytes, PPARγ is considered a master regulator of adipogenesis as it regulates fatty acid storage to decrease circulating levels of lipids and improve insulin sensitivity [77]. All three receptor subtypes have been identified in activated hepatic stellate cells, suggesting a possible role in fibrogenesis regulation and thus potentially modulated to address fibrosis present in severe NASH patients. The therapeutic precedent for agonists of these receptors has been conclusively established. Thiazolidinediones (TZDs), which predominantly target PPARγ, improve insulin sensitivity and have been used clinically to treat T2D patients. Fibrates, which predominately target PPARα, have been used in the clinic to treat various hyperlipidemias. Nevertheless, neither drug class is without safety issues. Some PPARγ agonists promote weight gain and have adverse effects on the cardiovascular system through a combination of cell-autonomous effects in cardiomyocytes via fatty acid oxidation [78] and indirect effects to increase salt and water retention. The increased risk of congestive heart failure with TZDs, which is likely due to fluid retention effects [79] despite no increased risk of death from cardiovascular causes [80], has restricted the clinical use of TZDs. Additional safety concerns with TZD therapy include increased fracture risk and partially disproven concerns about bladder cancers. Classical fibrates are weakly potent PPARα agonists that show only modest lipid-lowering efficacy relative to statins. Fibrates also demonstrate dose-related side effects from their underlying mechanism, including modulating cytochrome enzymes in the liver that can cause deleterious drug−drug interactions, limiting their utility in real-world practice. Notably, the fibrate gemfibrozil alters the hepatic metabolism of the statin cerivastatin, which can increase the risk of severe rhabdomyolysis [81]. Furthermore, fibrates have been associated with increased transaminase levels indicative of acute liver injury. Dual agonists that target PPARγ and PPARα, known as glitazars, were developed with the rationale of combining the insulin-sensitizing properties of PPARγ TZDs with the anti-fibrotic effects of PPARα fibrates. However, a number of these compounds reached the same fate as the TZDs based on clinical data showing increased cardiovascular risk [82] or renal failure risk [83]. These cumulative findings spurred discovery interest in higher-potency multi-agonists including differentially balanced co-agonists and pan-PPAR ligands aimed to capitalize on receptor signaling synergy, as well as the contrasting strategy of more selective PPAR ligands aimed to mitigate combinatorial side effects that may result from residual low-potency agonism at other PPAR isoforms.

The first PPARγ ligand to be studied in NASH patients was pioglitazone in the phase 2 PIVENS trial [84]. Pioglitazone is a TZD that is low to moderately potent and a semi-selective PPARγ agonist with approximately 7-fold less affinity to PPARα relative to PPARγ based on its initial pharmacological characterization [85]. Although pioglitazone failed to meet the primary objective of an improved NASH rate (34% vs 19% on placebo) as defined by improved hepatocellular ballooning without worsening of fibrosis and $\geq$3 NAS improvement, pioglitazone was associated with improved insulin sensitivity, reduced serum levels of ALT, and positive histological readouts on hepatic steatosis. This suggests the potential therapeutic utility of engaging PPARs, particularly PPARγ, to provide benefits on discrete aspects of NASH. However, body weight gain was evident in this trial, common to previous pioglitazone trials, and fibrosis improvement was not observed after 96 weeks of treatment. Nonetheless, the effects of pioglitazone on histological NASH endpoints seem more pronounced than with the more selective PPARγ agonist rosiglitazone in NASH patients [86,87], suggesting a contribution of PPARα activity to the differential efficacy of pioglitazone. It cannot be ignored that unlike rosiglitazone, pioglitazone lowers LDL cholesterol and triglycerides in diabetic patients [88], which likely contributes to the reduced progression of atherosclerosis and lower cardiovascular disease (CVD) risk associated with pioglitazone [89,90] and are components of an ideal drug candidate for NASH. Saroglitazar is a potent non-thiazolidinedione dual agonist of PPARα and PPARγ with selective preference for PPARα (approximately 5000-fold) [91]. Of note, saroglitazar is exquisitely potent at PPARα with subpicomolar potency (EC_{50} ~6.5 pM), whereas fenofibrate has double digit micromolar potency at PPARα (EC_{50} ~22.4 μM) [92]. In a 16-week global phase 2 trial in NASH patients (EVIDENCES IV; NCT03061721), 40.7% of patients on saroglitazar (4 mg) therapy achieved a $\geq$30% reduction in liver fat content vs 8.0% on placebo, and the mean liver fat content decreased by 3.9% relative to placebo [93]. Saroglitazar also significantly lowered ALT and triglycerides and improved insulin resistance as measured by HOMA-IR. More comprehensive clinical trials will hopefully provide direction to the potential of PPAR ligands with this balance toward PPARα and whether the amount of PPARγ activity in saroglitazar is ideally balanced for its benefits without liabilities.

Elafibranor is a dual PPARα and PPARδ agonist that has a ~10-fold preference for PPARα and a ~100-fold higher potency than the classical fibrate fenofibrate at PPARα. In a phase 2 trial in NASH patients (GOLDEN-505) [94], elafibranor did not meet the primary endpoint of NASH resolution without worsening of fibrosis nor was a difference observed in the key secondary endpoint of fibrosis improvement. A post hoc analysis suggested differential efficacy upon patient stratification, which prompted additional clinical study. Results from the interim analysis of the RESOLVE-IT phase 3 trial in NASH patients with fibrosis stages 2−3 (NCT02704403) suggested that elafibranor (120 mg) again did not meet the predefined primary surrogate efficacy endpoint of NASH resolution without worsening of fibrosis (19.2% of patients on elafibranor vs 14.7% on placebo) nor was the secondary endpoint improvement of fibrosis met with elafibranor after 72 weeks (24.5% with elafibranor vs 22.4% with placebo). However, decreased plasma triglycerides and ALT were observed [95]. Nonetheless, these data do not support ongoing clinical evaluation of elafibranor in NASH.

Lanifibranor (IVA337) is a moderately potent imbalanced pan-PPAR agonist that favors PPARγ by fourfold over PPARδ and seven-fold over PPARα [96]. In a 24-week phase 2 trial (NATIVE) in NASH patients, 45% of patients achieved NASH resolution without worsening of fibrosis on lanifibranor therapy at a daily dose of 1200 mg/day vs 19% of patients on placebo [97]. The proportion of patients on high-dose lanifibranor who achieved $\geq$1 stage improvement in fibrosis without worsening of NAS was 42%, whereas 24% of patients in the placebo group achieved this endpoint. Decreases in ALT, triglycerides, and insulin were also evident with lanifibranor therapy. Body weight increased by 3.1% from baseline and the incidence of peripheral edema increased relative to placebo (8.4% vs 2.5%). It will be important to know if this pan-PPAR agonist differentiates from PPARγ selective agonists such as pioglitazone with respect to effects on body weight or cardiovascular function in longer-term trials.

As opposed to the aforementioned PPAR ligands with varying degrees of activity at multiple receptors, pemafibrate is a selective PPARα agonist with higher potency relative to classical fibrates [98]. Pemafibrate is currently in development for atherogenic dyslipidemia but may have utility in NASH. In clinical evaluations, pemafibrate showed greater efficacy to lower triglycerides and ALT than fenofibrate in patients with atherogenic dyslipidemia, albeit with less effect on serological cardiorenal biomarkers [99−101]. This recapitulates the effects seen with classical fibrates yet the effect sizes appear larger, suggesting that improved PPARα can drive differential efficacy with

respect to reducing atherogenic dyslipidemia, which is an important characteristic of any metabolically targeted therapeutic for NASH. Whether the precise activity at PPARα is efficacious enough to improve histological features of NASH is unknown.

Seladelpar is a potent and selective PPARδ agonist that showed benefits in a clinical trial for cholangitis [102], but safety concerns regarding atypical histology was observed although these were not believed to be treatment related. Furthermore, in a 52-week phase 2 trial in NASH patients, seladelpar (50 mg) only trended to improve NASH resolution (26%) and fibrosis (37%) vs placebo (8% and 20%, respectively) without any noticeable effect on liver fat fractions [103]. Although the efficacy of this selective PPARδ on NASH resolution and hepatic fat content appeared less than what was reported with the pan-PPAR agonist lanifibranor, the effect on fibrosis improvement suggests that PPARδ activity has the potential to address this important component of NASH pathology despite a lack of this efficacy observed with the PPARδ/PPARα dual agonist elafibranor.

There is substantial nuance to the pharmacological profile of PPAR agonists that arises from the pleotropic actions of each individual receptor isoform, the different selective pressures on each individual receptor for each unique molecule, and varying tissue distribution patterns of the receptors and ligands. Therefore, indirect comparisons between them can be problematic. Regardless, as NAFL and NASH can be considered multi-organ diseases resulting from reciprocal dysfunction in other metabolically active endocrine organs, the potential to address extra-hepatic dysfunctions in metabolic syndrome is appealing [104]. A finely tuned pan-PPAR agonist may hold the most promise of all hormonal-based therapies because of these systemic and diverse mechanisms. Further supporting this high potential, finely tuned pan-PPAR agonists have the potential to address all three hepatic pathophysiological pathways of NASH (metabolism, inflammation, and fibrosis) by mechanisms within the three main hepatic cell types (hepatocytes, stellate cells, and Kupffer cells). Striking the right balance in PPAR isoform selectivity and agonism is critical for optimizing the therapeutic index, notably to limit adverse effects on the cardiovascular system while maximizing systemic efficacy. However, due to the heterogeneity of NASH, the optimum PPAR profile and balance may differ considerably from one patient to the next. Thus, a personalized or precision medicinal approach has appeal but the static nature of small molecules of a fixed PPAR balance may preclude this. However, the judicious combination of select mono-agonists seemingly has substantial upsides. The clinical data suggest that PPAR ligands that target individual receptors have limited overall efficacy in NASH as described herein. However, the precise combination of selective PPAR ligands as well as agonists for other nuclear hormone receptors such as FXR and THR-β has considerable upside to meet individual patient needs. To facilitate this, continued drug discovery efforts must be integrated with exhaustive genotypic and phenotypic characterization of NASH patients so that the discovery of biomarkers that predict drug success can enable tailored treatment algorithms.

3. GASTROINTESTINAL AND NEUROENDOCRINE PEPTIDE HORMONES

The gastrointestinal tract and liver are interconnected components of the entero–insular axis and bidirectionally modulate reciprocal function via various hormonal cues mediated by circulatory conduits such as the biliary tract and portal vein [105]. These hormonal cues that modulate liver cell function include peptides endogenously secreted from intestinal enteroendocrine cells and the endocrine pancreas, but also involve paracrine and autocrine actions of hepatokines. Integrative neuroendocrine circuitries connect the central nervous system, notably the hypothalamus, and the liver. Hepatic function can be modulated by neurosecretory hormones in response to various afferent cues originating in the periphery and central neurotransmitter input. These neurosecretory hormones, of which many are peptide-based hormones, can either directly act at the liver or indirectly through a cascade of subsequent endocrine signals originating from upstream sources. Drug candidates for T2D and obesity that are based on these peptide hormones are continually expanding and many have shown unprecedented therapeutic potential for metabolic diseases and other endocrine disorders. Peptide hormones that have shown the most therapeutic potential include growth hormone secretagogues, fibroblast growth factors, and incretin members of the glucagon superfamily of peptides.

3.1. Growth hormone-releasing hormone

Growth hormone-releasing hormone (GHRH) is an endocrine hormone produced in the hypothalamus and works on its receptor (GHRH-R) in the anterior pituitary to stimulate the release of growth hormone (GH). GH subsequently can engage hepatocytes to produce insulin-like growth factor-1 (IGF-1) and induce lipolysis in adipocytes via promotion of hormone-sensitive lipase. The GHRH analog tesamorelin, which is modified with a hexanoyl moiety at the N-terminus to improve proteolytic stability, has shown benefits in HIV patients with lipodystrophy and GH deficiency to reduce circulating triglycerides and visceral adipose fat [106] and decrease in serum levels of ALT [107]. In HIV patients with NAFLD, tesamorelin (2 mg daily) caused a greater reduction in relative hepatic fat fraction (37%) relative to placebo (therapy group decreased by 32%, whereas a placebo group gained 5%) [108]. As expected, IGF-1 levels increased and visceral adipose tissue decreased, but circulating triglycerides trended to increase after 12 months of treatment. An analysis of biopsied livers demonstrated that tesamorelin increased transcriptional markers of oxidative phosphorylation and decreased gene sets linked to inflammation [109]. Whether these benefits on hepatic fat content translate to non-HIV patients remains to be proven, but recent clinical results with GH in obese NAFL patients [110] lend credence to targeting this biological pathway for NASH benefits. The recently announced phase 3 trial of tesamorelin in general NASH patients will hopefully provide definitive proof. However, the hyperglycemic propensity of GHRH action must be considered when testing in T2D patients or fragile NASH patients as these individuals are predisposed to glycemic derangements. Another consideration for GHRH-based therapy for NASH is that although GH response to GHRH is higher in women than men [111], postmenopausal women have dampened GH secretion following GHRH stimulation [112]. This is important since the prevalence and incidence of NAFLD are higher in men than premenopausal women, yet these trends normalize in postmenopausal women [113]. This suggests that GHRH-based therapies may be more effective if segmented for premenopausal women.

3.2. Fibroblast growth factors

Fibroblast growth factors 21 (FGF21) and FGF19 are endocrine hormones with pleiotropic actions to regulate systemic energy metabolism and lipid homeostasis. FGF21 activates a plasma membrane receptor complex that consists of canonical FGF receptors and a β-klotho co-receptor (Klb). FGF21 primarily activates FGFR1c, which is predominantly expressed in adipocytes, but also has activity at FGFR2c and FGFR3. FGF19 also requires the Klb co-receptor to activate canonical

FGFRs and principally activates FGFR4, which is predominantly expressed in hepatocytes. FGF19 regulates bile acid synthesis by decreasing CYP7A1 levels, which is the rate-limiting step in converting cholesterol to bile acids. FGF19 and FGF21 exert insulin-like actions on glucose and lipid homeostasis through direct and indirect actions on hepatocytes. By nature of its action via hepatic FGFR4, the therapeutic potential of FGF19 is limited because of a correlation of increased circulating levels with hepatic mitogenicity in rodents [114]. Alternatively, exogenous administration of FGF21 analogs has shown robust preclinical efficacy to improve hyperglycemia, dyslipidemia, and body weight in rodents. However, these effects on glycemia and body weight have not translated to humans with protype FGF21 analogs [115]. Despite this, the robust and early in-treatment effect to lower triglycerides has largely translated in humans for these first-generation analogs [116]. Medicinal chemistry efforts for optimizing FGF21 focused on protracting time action by site-specific mutagenesis, conjugation of high-molecular-weight moieties, or increasing selectivity toward the FGFR1c/β-Klotho complex. As for the latter, a bispecific antibody (NGM313; now MK3655) has been reported but uncertainty remains with solely engaging the FGFR1c/β-Klotho complex and the resultant maximal efficacy *in vivo*. FGF21 is capable of activating three FGFR isoforms (1c, 2c, and 3c) in tandem with β-Klotho [117], but FGFR1c appears to be necessary and sufficient for adipocyte-centric metabolic actions of FGF21 in preclinical models [118], although the contributing effects of FGFR2c and FGFR3c to the liver-centric mechanisms of FGF21 have not been exhaustively studied.

Aldafermin (NGM282), an optimized FGF19 analog with key mutations that essentially eliminate its mitogenic liability, is in development for NASH. Aldafermin is modified in its N-terminal region relative to native FGF19, including deletion of a 5 residue stretch along with 3 point mutations [119]. Together, these changes enable biased FGF receptor signaling away from STAT3 activation, and this differential downstream signaling is suggested to impart protection from hepatocellular mitogenicity [120]. At week 24 in a phase 2 clinical trial in patients with biopsy-confirmed NASH and fibrosis stages 2−3, aldafermin (1 mg) lowered the relative liver fat content by 26% (placebo-corrected), and 66% of patients achieved a ≥30% reduction in relative liver fat content on aldafermin vs 29% of patients on placebo [121]. An improvement of at least a single stage in fibrosis without worsening of NASH was reported in 38% of patients on aldafermin therapy vs 18% on placebo. NASH resolution without worsening of fibrosis was achieved in 24% of patients on aldafermin therapy vs 9% on placebo. Serum levels of ALT were reduced by 43% by aldafermin relative to placebo. PRO-C3 levels were likewise reduced with aldafermin therapy. As a potential drawback, LDL cholesterol levels were increased by aldafermin treatment but not surprising based on its mechanism. These increased levels of LDL cholesterol were effectively managed by concomitant statin treatment, which resulted in 96% of patients in the aldafermin group ultimately receiving statin therapy vs 36% of patients in the placebo group. A similar combination strategy of statin therapy to offset LDL cholesterol increases evident with FXR agonism has yet to be explored, but drug−drug interactions must be carefully evaluated. Serum triglycerides were also reduced with aldafermin treatment. The improvement in composite endpoints of fibrosis, NASH resolution, and hepatic fat irrespective of the baseline fibrosis stage as advanced as F3 suggests the ability to target more severe NASH patients, something that may provide a competitive advantage for aldafermin relative to other therapeutics targeting metabolic mechanisms.

With respect to targeting the FGF21 pathway, pegbelfermin (BMS-986036) is a long-acting FGF21 analog conjugated to a polyethylene glycol polymer (PEG). In a phase 2 trial in obese T2D patients, pegbelfermin decreased levels of PRO-C3 and serum triglycerides and increased HDL cholesterol and adiponectin [122]. In a phase 2 trial in patients with NASH, daily doses of 10 mg of pegbelfermin decreased relative hepatic fat content by 31.6% (placebo-corrected) after 16 weeks of treatment, and 56% of patients achieved a ≥30% reduction in relative liver fat fractions vs 24% on placebo [123]. Pegbelfermin also decreased levels of ALT, PRO-C3, and triglycerides. Liver biopsies to determine histological improvement were not performed in this study so additional research is required to understand the potential for NASH. Another protracted FGF21 analog, BIO89-100, is glyco-PEGylated with a pharmacokinetic profile that may support every other week dosing in humans and recently showed favorable effects to lower relative hepatic fat content with various dosing regimens [124]. This affords a competitive advantage vs pegbelfermin despite more complicated manufacturing considerations for glyco-PEGylation. Questions remain about the eventual accumulation of PEG that is deposited in already diseased hepatocytes, but there have not been signs of overt toxicity in these short-term clinical studies.

AKR-001 (efruxifermin), formerly AMG876, is a long-acting FGF21 analog with three key mutations fused to the Fc domain of human immunoglobulin 1. Efruxifermin is an Fc-conjugate dimer and is less potent in activating FGFR-Klb complexes than native FGF21. The protein backbone of efruxifermin features three mutations (Leu98Arg, Pro171Gly, and Ala180Glu; RGE) that collectively improve aqueous formulation stability, reduce proteolytic degradation to enhance *in vivo* duration of action, and enhance the functional potency of the FGFR-Klb-binding interaction [125], which presumably affords more potency and a longer half-life relative to pegbelfermin. In a 4-week multiple-ascending dose trial in which T2D patients received weekly doses of efruxifermin from 7 mg to 140 mg [126], efruxifermin showed positive trends to decrease atherogenic lipid species, including triglycerides, non-HDL cholesterol, and apolipoprotein B. With 70 mg of daily dosing of efruxifermin for 3 weeks (equivalent to 3 doses of weekly efruxifermin), serum levels of triglycerides decreased by 69% (placebo-corrected) from baseline and serum levels of non-HDL cholesterol reduced by 30% from baseline (placebo-corrected). Unlike other experimental treatments in clinical development for NASH, including aldafermin [127] and the FXR agonist obeticholic acid [62], reduced bile acid synthesis and subsequent increased serum LDL cholesterol was not observed with efruxifermin. Importantly, whereas measurable efficacy to lower triglycerides and non-HDL cholesterol was observed at 21 mg following daily dosing, efficacy with respect to glycemic markers and improved insulin sensitivity were only observed at higher doses. Modest tachycardia (2−3 beats per minute) at the maximally efficacious dose of 70 mg daily was observed.

Efruxifermin (28 mg, 50 mg, and 70 mg) is currently being investigated in a 12-week phase 2a trial in patients with biopsy-confirmed NASH and fibrosis stages 1−3 (BALANCED; NCT03976401). Interim results were recently released and demonstrated that all dose levels of efruxifermin met efficacy endpoints on hepatic fat content at 12 weeks [128]. Efruxifermin caused a dose-dependent decrease in placebo-corrected relative hepatic fat content (−63.0% at 28 mg, −71.0% at 50 mg, and −72.0% at 70 mg). Furthermore, across the efruxifermin dose groups, a greater proportion of patients achieved at least a 30% reduction in liver fat relative to placebo (75−85% for efruxifermin and 10% for placebo). Significant reductions in serum levels of ALT, PRO-C3, non-HDL cholesterol, and triglycerides were also evident across the efruxifermin dose groups, and body weight loss (−3.7 kg), HOMA-IR improvement (−49%), and HbA1c lowering (−0.5%) were observed for the highest dose level. Of the treatment responders who

had end of treatment biopsies across all of the dose groups, 48% of patients achieved NASH resolution without worsening of fibrosis, 48% of patients achieved at least a one-stage improvement in fibrosis without worsening of NASH, and 28% achieved a two-stage improvement. However, only two patients from the placebo group were analyzed, so caution must be taken when comparing the efficacy to other phase 2 trials. Because of the effects of AKR-001 on improved insulin sensitivity, decreased atherogenic lipids, and reduced hepatic fat content, these pleiotropic actions give efruxifermin the potential to lower patient risk of major cardiovascular events, which is a considerable upside for this class of therapeutics.

Overall, biochemical engineering approaches to mitigate the limitations of first-generation analogs within the FGF family have been largely successful in translating to differential efficacy in early clinical evaluations. It is clear from early results that this family of therapies represents a viable therapeutic mechanism that is well tolerated for treating atherogenic dyslipidemias, hepatic steatosis, and associated diseases with a mechanistic upside to provide long-term benefits to lower cardiovascular disease risk. The unintended increase in LDL cholesterol for FGF19-based therapies, the unknown effects on fibrosis, and the heightened dose requirement to influence hyperglycemia of FGF21-based therapies must be considered when evaluating the ultimate potential of this drug class.

3.3. Incretin receptor agonists

Glucagon-like peptide 1 (GLP-1) is a gastrointestinal hormone that signals through receptors (GLP-1R) that are predominantly expressed in pancreatic islets and the central nervous system (CNS). Islet actions of GLP-1 promote glucose-sensitive insulin secretion to regulate glucose homeostasis and central actions of GLP-1 promote anorectic actions to regulate systemic energy balance. Numerous GLP-1R agonists have shown therapeutic benefits to improve body weight and glycemic control in T2D patients with next-generation long-acting molecules showing differential efficacy and lessened risk of cardiovascular events [24,129,130]. Preclinical data [131,132] and emerging evidence in clinical studies suggest that GLP-1R agonists also have benefit in NAFL and NASH, although the mechanisms governing these liver benefits have not been crystalized and could be a secondary benefit of the improved body weight and glycemic control afforded by these therapies. Although most evidence points to a lack of GLP-1R expression in hepatocytes, debate remains whether GLP-1R is expressed directly in hepatocytes [133].

Liraglutide is a GLP-1R agonist modified with a C16 fatty acid that is suitable for once-daily administration in humans. Lower doses of liraglutide are indicated for T2D, whereas higher doses are indicated for obesity. In a 48-week phase 2 trial in overweight patients with NASH [134], 39% of patients who received liraglutide (1.8 mg; T2D dose level) showed NASH resolution without worsening of fibrosis compared to 9% on placebo. Only 9% of patients who received liraglutide showed progression of fibrosis vs 36% of patients on placebo. A greater proportion of patients in the liraglutide group showed improved steatosis and hepatocyte ballooning vs placebo, but lobular inflammation and composite NAS did not improve. Serum levels of ALT were unchanged at the end of treatment but appeared to decrease over time. ELF markers and GGT were improved with liraglutide therapy. After 48 weeks, liraglutide lowered body weight by 4.2% and HbA1c by 0.5% relative to placebo, and a post hoc analysis suggested that body weight loss and metabolic improvements contributed to the improved liver histology, but interestingly were not likely the sole contributor. Higher doses of liraglutide, notably the 3.0 mg dose indicated for obesity, were not approved for use in humans at the time of this trial but it would be intriguing to know if increased efficacy could have been achieved with this higher dose.

Semaglutide is a GLP-1R agonist modified with a C18 diacid-based fatty acid that is suitable for once-weekly subcutaneous administration in humans and as a once-daily oral formulation [135]. Semaglutide has demonstrated differential efficacy to lower body weight in obese and T2D patients relative to best-in-class compounds including liraglutide and dulaglutide [130,136]. In a 72-week phase 2 trial in patients with NASH and fibrosis stages 1–3, recently disclosed data showed that a greater proportion of patients on daily doses of semaglutide (0.1–0.4 mg) achieved resolution of NASH without worsening of fibrosis vs placebo irrespective of T2D status. Of note, 58.9% of patients who received 0.4 mg daily doses of semaglutide achieved NASH resolution without worsening of fibrosis vs 17.2% of patients on placebo [137]. However, fibrosis without worsening of NASH was not improved with semaglutide treatment relative to placebo despite estimates of liver stiffness showing improvement. Select serological measures also improved with the highest dose of semaglutide relative to placebo, including ALT and triglycerides. Body weight loss was likely a substantial contributor to the efficacy observed with semaglutide as a similar dose caused ~15% body weight loss after 52 weeks in obese patients [24]. In the trial of NASH patients, semaglutide (0.4 mg) decreased body weight by 12.6% from baseline relative to 0.6% in the placebo group.

GLP-1R-based multi-agonism is a growing therapeutic strategy with the goal of enhancing efficacy while minimizing dose-dependent side effects. Peptides with integrated co-agonism for GLP-1 and glucose-dependent insulinotropic polypeptide (GIP) have shown enhanced metabolic efficacy in preclinical models [138,139] and recently in emerging clinical data [140,141]. Similar to GLP-1, GIP is secreted from the gut in response to nutrient stimuli. The GIP receptor (GIPR) is predominantly expressed in pancreatic islets but also in the CNS and adipocytes. The primary physiological role of GIP is to promote glucose-stimulated insulin secretion and alter adipocyte lipid metabolism. Of note, a retrospective analysis of the ADDITION-PRO trial showed a correlation of GIP levels with lower LDL cholesterol [142]. The first GLP-1/GIP co-agonist to advance to a clinical trial (MAR709; NNC0090-2746) was a balanced GLP-1/GIP co-agonist acylated with C16 fatty acid. In a 12-week phase 2a trial, patients who received MAR709 showed improved measures of glycemic control and trends for decreased body weight and lipids, including total cholesterol [140]. Tirzepatide is a once-weekly injectable GLP-1/GIP co-agonist (imbalanced to favor GIP activity) acylated with a C20 diacid-based fatty acid that is currently in development for T2D and being explored for obesity and NASH. In a 26-week phase 2 trial in T2D patients [141], the highest dose of tirzepatide (15 mg) decreased HbA1c by 2.5% and lowered body weight by 12.2% (11.3 kg from baseline) relative to placebo. In post hoc analyses [143], higher doses of tirzepatide decreased NASH-related biomarkers, including ALT and PRO-C3, and increased adiponectin, but it is unknown how many patients had NASH nor was liver fat content measured. Furthermore, it is unknown how much of these improvements in liver biomarkers were a result of the substantial body weight loss. A phase 3 trial to study tirzepatide in NASH patients is planned (SYNERGY-NASH; NCT04166773). Although the precise mechanisms of GIP pharmacology that lead to metabolic efficacy have yet to be proven, the effects are likely mediated by improved adipocyte lipid handling, increased insulin sensitivity, and CNS-driven anorectic actions [144].

Glucagon is a peptide hormone secreted from pancreatic islets in response to low glucose and signals through glucagon receptors (GcgR) that are primarily expressed on hepatocytes to increase glucose

output. Glucagon actions at the level of hepatocytes also include regulation of lipogenic machinery and cholesterol biosynthesis. Therefore, integration of glucagon activity in multi-agonists may provide a direct cell-autonomous effect at the level of hepatocytes and provide the potential to differentiate on hepatic outcomes. Peptides with integrated co-agonism for GLP-1 and glucagon [145—147] or triple GLP-1/GIP/glucagon receptor agonism [148] have also shown enhanced metabolic efficacy in preclinical models of obesity, notably marked improvements in hepatic steatosis, lipid serology, and body weight relative to GLP-1R mono-agonists. GLP-1/glucagon co-agonists have recently shown improved histological markers in mouse models of NASH [149,150].

Cotadutide (MEDI0382) is an acylated (C16) once-daily injectable GLP-1/glucagon co-agonist currently in development for NAFL and NASH. Cotadutide is imbalanced by approximately 5-fold to favor GLP-1R potency relative to GcgR. In a 26-week phase 2 trial in overweight or obese patients [151], cotadutide (100—300 μg) caused dose-dependent reductions in body weight and HbA1c relative to placebo. At a 200 μg dose, body weight loss and HbA1c improvement were equivalent to those of a liraglutide (1.8 mg) comparator. Importantly, with similar body weight loss, cotadutide caused greater reductions in ALT than liraglutide, demonstrating that glucagon action can differentiate vs GLP-1R mono-agonism with respect to liver injury markers. At the highest cotadutide dose level (300 μg), greater body weight loss was achieved than liraglutide, and interim analyses suggested significant decreases in non-invasive liver biomarkers of fibrosis (NFS and FIB-4) and LDL cholesterol were observed with cotadutide treatment relative to placebo [152]. As glucagon has chronotropic effects, it is important to note that the increase in the heart rate (∼2.5 bpm) was consistent with the increase observed with liraglutide. The question remains whether compounds with greater relative GcgR potency would provide further differentiation regarding liver and body weight efficacy without sacrificing the therapeutic index, particularly with respect to glycemic control and cardiovascular effects. Nonetheless, these data support prospective clinical trials with cotadutide and other glucagon-based agonists in patients with NASH.

HM15211 is an Fc-conjugated GLP-1/GIP/glucagon triple agonist with an undisclosed potency ratio. In a phase 1b/2a clinical trial in non-diabetic obese patients with NAFLD, HM15211 dose-dependently lowered body weight and relative hepatic fat content. After 12 weeks, HM15211 lowered relative hepatic fat content by as much as 88% and body weight by as much as 5.1% relative to placebo controls [153]. For HM15211 and other tri-agonists of this nature, the most important factor that will likely cause differentiation in the class is the ideal balance of activities across the three receptors. The nature of the protracting moiety should also be considered as it can greatly influence biodistribution such that the *in vivo* receptor balance would differ from the balanced measured in isolated *in vitro* systems as was recently suggested from quantitative assessments of *in vivo* receptor occupancy of incretin multi-agonists [154].

4. FUTURE PROSPECTIVE COMBINATIONS

Evidence from these proof of concept clinical trials demonstrated that specific features of NASH are responsive to pharmacological intervention; however, a majority of patients in these trials did not show improvements in the two endpoints mandated by the US FDA to be assessed in late-stage clinical studies: 1) NASH resolution with no worsening of fibrosis and 2) improvement of fibrosis with no worsening of NASH. Fibrosis stage is the strongest predictor of mortality in patients with NAFLD, and FDA guidance in treatments for NAFLD stresses the importance not only of liver fat reduction, but also preventing fibrosis and reducing inflammation [155]. The cumulative evidence from these clinical trials in NASH patients offers optimism to improve patient outcomes, and although they may ultimately result in regulatory approval as a long-term monotherapy, the effect sizes and patient proportion data are modest. To maximize efficacy and minimize toxicity of these metabolism-focused drugs, tissue-specific agonists, partial receptor agonists, and biased signaling agonists may ultimately be required to sufficiently improve the therapeutic index, but these types of molecules are largely discovered by serendipity rather than rational design. Although these metabolism-specific drug candidates have been shown to be able to prevent the progression of fibrosis, current evidence suggests that simply targeting the dysregulated metabolic milieu may not have enough efficacy to reverse established fibrosis. Since NAFLD is a multi-factorial spectrum disease, judiciously selected combinations and compounds with multi-modal receptor function targeting different pathogenic nodes of NAFL and NASH may provide the differential performance needed. Comparative evidence across the NASH trials studying various PPAR ligands indeed suggested differentiation capability with semi-selective or pan-PPAR agonists relative to compounds acting through a single receptor subtype, thus providing pseudo proof of concept that targeting multiple pathways can provide synergistic efficacy while minimizing side effects.

Increasing discovery and developmental efforts are dedicated to studying combination therapies. At least 10 combinations are already in clinical evaluation or progressing to clinical trials, and mostly involve combination of a metabolism-focused agent with an agent geared toward inflammation or fibrosis [156]. However, there can be substantial potential in combining compatible metabolic-targeted therapeutics that have complementary non-redundant mechanisms such as GLP-1R agonists, thyromimetics, and FXR ligands. As discussed, NAFL and NASH are heterogenous diseases. Despite similar histological manifestation of the diseases, the pathogenic process to reach the clinical representation can drastically differ from patient to patient. Moreover, the possibility of drug combinations as the preferred future therapeutic option is growing in prominence because of concern that engaging a single target and pathology will not be sufficiently potent over the long term and may not be broadly applicable across diverse patient populations. Therefore, rather than a one-size-fits-all approach to treating this disease using a single molecule or fixed combination, therapeutic strategies with the agility to select and modify different combination partners and their dosages that are tailored by patient-specific meta-data may be where most unmet patient needs can be addressed.

4.1. Single molecule combinations of nuclear hormones and peptide hormones

Pioneering work in oncology combinatorial therapy research, specifically antibody—drug conjugates as precision medicines, has inspired the pursuit of similar strategies for metabolic diseases. The strategy is to target nuclear hormones to specific tissues via a pharmacologically active peptide carrier to impart tissue bias and multi-functional activities within a single molecule. Single molecule strategies that have been explored include GLP-1 mediated delivery of estrogen [157], dexamethasone [158] or oligonucleotides [159], neuropeptide Y-mediated delivery of PPAR ligands [160], and glucagon-mediated

delivery of thyroid hormone [161]. The latter combination was particularly designed to capitalize on the combined and complementary effects of glucagon and thyroid hormone to decrease hepatic fat content and circulating levels of atherogenic lipids with the potential to treat dyslipidemia, NAFL, and NASH.

Glucagon-mediated targeting of thyroid hormone offers an alternative to designing isoform-selective thyromimetics, and unlike other hepatic targeting strategies, glucagon-mediated targeting mimics a Trojan Horse approach with inherent dual pharmacology. The integrated co-agonism supports coordinated poly-pharmacology arising from the targeted thyroid hormone payload and activity within the glucagon carrier. It was shown that by leveraging glucagon as a targeting ligand, thyroid hormone action can be enriched in the livers of rodents where complementary mechanisms that affect lipid and cholesterol metabolism are engaged. These metabolic actions were largely uncoupled from deleterious effects on the cardiovascular system, notably a lessened but not totally cancelled hypertrophic effect on the heart and minimal influence on left ventricular function. This was presumably through glucagon-governed biodistribution that enriched T_3 deposition in the liver and largely spared cardiomyocytes from direct thyroid hormone action that would happen with untargeted T_3 action. Similarly, glucagon-mediated targeting of thyroid hormone action should partially spare bio-delivery to bone and minimize deleterious effects on bone metabolism. A negative effect on bone density was only observed in mice following treatment with supra-pharmacological doses of glucagon/T_3 conjugate. Despite aspects of thyroid hormone action that exacerbate insulin resistance [162], hepatic-directed thyroid hormone action prevented the hyperglycemic propensity of glucagon, thus causing a reciprocal mitigation of inherent liabilities of each individual component in these rodents. This glucagon/T_3 conjugate effectively lowered body weight by increasing energy expenditure and lessened hepatic fat content in mice fed obesogenic diets. Furthermore, the conjugate lowered circulating triglycerides and total cholesterol and prevented the accumulation of atherosclerotic plaques in the aortic root of Western diet-fed LDLR−/− mice. Importantly, the glucagon/T_3 conjugate lowered and improved hepatic steatosis and the mild fibrosis evident in mice fed a NASH-inducing diet. Collectively, these findings were the basis for a dedicated drug discovery and development program focused on optimizing the chemical structure to maximize the therapeutic window.

Advancing these provocative preclinical findings into a drug development candidate has proven challenging with respect to precisely quantifying the improved therapeutic index using *in vitro* and *in vivo* preclinical models. At the time of the original publication, it was recognized that the prototype conjugate may require chemical optimization with more rigorous stability and toxicity profiling before advancing through development. It was discovered that the prototype glucagon/T_3 conjugate, which contained a relatively simple γ-glutamic acid-based covalent linker between the modified glucagon backbone and conjugated T_3 moiety, was partially hydrolyzed to release T_3 when spiked into mouse-derived plasma and with extended incubation. Importantly, this conjugate linker was completely stable when spiked into human plasma. It is well known in the pharmaceutical industry that mouse plasma has a greater propensity to metabolize and degrade compounds than human plasma. However, the challenge is that rodents were identified as one of the species for toxicity assessment, but artificial toxicity readouts could be expected since conjugate stability has seminal importance for assessing an improved therapeutic index. Nonetheless, an improved therapeutic index in rodents was clear with the prototype conjugate, but the magnitude of the improvement was not enough for these mice and less than what was originally estimated after thorough assessment. With extended dosing durations at higher doses, a hypertrophic effect on the heart was evident at efficacious doses. Formal pharmacokinetic studies in mice proved that the T_3 moiety was indeed liberated from the conjugate to reach exposure levels that were seemingly responsible for the heart weight increase. By pharmacodynamic and pharmacokinetic comparison to related analogs in mice, it was concluded that the efficacy on body weight and lipid metabolism was predominantly governed by the intact conjugate, thus providing further proof of concept. Another confounding factor that challenged the interpretation of historical data and plans for toxicity research was the discovery that GcgR tissue expression patterns, particularly within the cardiovascular system, are different in rodents vs humans [163]. In addition to the hydrolysis of the covalent linker in mouse plasma, additional proteolytic processing was observed in the glucagon backbone utilized. The sequential decomposition of the glucagon backbone seemed to exacerbate the hydrolysis of the linker to T_3. Therefore, it was recognized that to support preclinical and non-clinical development, the protype structure required optimization to mitigate these biological instabilities, and that more sophisticated *in vitro* models need to be developed to accurately assess the structure—activity relationship and optimize chemical structures. Advanced cellular models to study GcgR-dependent cellular uptake and thyroid hormone action were generated and paired with quantitative drug metabolism profiling. These refined *in vitro* and *in vivo* models have guided the chemical evolution of the prototype conjugate in which the structure—activity relationship focused on fine-tuning the correct meta-stability into the linker. As with most unimolecular multifunctional agonists, fine-tuning the relative constituent activity is of the utmost importance to maximize efficacy and a focus of ongoing chemical optimization. Results of these lessons and advances will be communicated shortly.

5. CONCLUSION

The alarming rise in the worldwide prevalence of T2D, obesity, and dyslipidemia has been accompanied by an increase in NASH such that 100 million cases are projected in the US alone by 2030 [164]. The fatal outcomes of NASH include cirrhosis, liver failure, and hepatic malignancies as well as extrahepatic comorbidities including cardiovascular disease. Although worldwide patient population characteristics can differ, a large majority of NAFL and NASH patients are overweight or obese in the US and Europe [165]. It is known that significant weight loss, regardless of the method by which it is achieved, will have a beneficial effect on NASH. It has been shown that that sustained weight loss of 10% is associated with a reduction in liver fibrosis [19], bariatric surgery provides long-term resolution of NASH and fibrosis regression [29], and meta-analysis of the NASH clinical trials shows an association between weight loss and biomarkers indicative of improved liver health [22]. As next-generation T2D and obesity medicines are now achieving $>$10% body weight loss and approaching 20% in clinical trials, we are on the right trajectory for developing effective and perhaps transformational medicines for NAFL and NASH. However, the inherent complexity of the diseases warrants the necessity of multiple options to select from when tailoring a precision treatment regimen for patients. Downstream efforts to identify a constellation of biomarkers that reliably predict disease status and pathogenesis will streamline clinical evaluations and support standardization of clinical trial designs. Furthermore, studying new therapies in rare cholestatic liver diseases such as

primary biliary cholangitis and primary sclerosing cholangitis will allow earlier proof of concept assessment in humans. Continued upstream work to advance translatable *in vitro* cell models, such as hepatic cell organoids [3], and *in vivo* preclinical models, such as Gubra-Amylin NASH DIO mice [4], will aid more in-depth disease understanding and support screening of novel pharmacotherapies. Together, these systematic efforts will catalyze the discovery of new therapeutic strategies with increased innovation so that transformative medicines can be effectively and efficiently developed.

CONFLICTS OF INTEREST

Brian Finan, Sebastian D. Parlee, and Bin Yang are current employees of Novo Nordisk and are inventors of some of the intellectual property discussed herein. Novo Nordisk did not fund any work pertaining to this manuscript. The opinions discussed herein are those of the authors and not necessarily those of Novo Nordisk.

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