NASH Drug Treatment Development: Challenges & Lessions

Herbert Tilg*[1], Christopher D. Byrne*[2], Giovanni Targher*[3]

1) Department of Internal Medicine I, Gastroenterology, Hepatology, Endocrinology & Metabolism, Medical University Innsbruck, Austria
2) Southampton National Institute for Health and Care Research Biomedical Research Centre, University Hospital Southampton and University of Southampton, Southampton, UK
3) Department of Medicine, Endocrinology and Metabolism, University of Verona, Verona, Italy

*All authors contributed equally

Word count main text: 5467. References: 124

Contributors section: All authors contributed equally

Correspondence to:
Herbert Tilg, M.D., Department of Internal Medicine I, Medical University Innsbruck, Innsbruck, Austria
Phone: +43 512 504 23539, Fax: +43 512 504 23538
E-mail: herbert.tilg@i-med.ac.at

Abstract

Non-alcoholic fatty liver disease (NAFLD) has become the commonest chronic liver disease worldwide. Although NAFLD is tightly linked to obesity and type 2 diabetes mellitus, this liver disease also affects lean subjects. NAFLD increases the risk of developing cardiovascular disease, chronic kidney disease and certain extrahepatic cancers. There is currently no licensed pharmacotherapy for NAFLD, despite numerous clinical trials in the last two decades. Currently, we do not understand why so many drugs have failed and it is debatable whether all targeted mechanisms were appropriate or defined trial endpoints were too ambitious. Since cardiovascular disease is the predominant cause of mortality in people with NAFLD, future pharmacotherapies for NAFLD must take into account associated cardiometabolic risk factors. The successful use of glucose-lowering drugs used in the treatment of type 2 diabetes mellitus in patients with NAFLD indicates that this strategy is important and is worth developing further. Public awareness of NAFLD needs to increase since collaboration between all stakeholders is vital to enable a holistic approach to the successful treatment for NAFLD. (172 words)

Search strategy and selection criteria

References for this clinical narrative Review were identified by the authors through searches of PubMed with the search terms “NAFLD” OR “non-alcoholic fatty liver disease” OR “non-alcoholic steatohepatitis” OR “NASH” OR “fatty liver” AND “treatment” OR “pharmacotherapy” OR “clinical trials”. We have searched up to March 31, 2023. We have considered the relevant literature cited in these papers. Only articles published in English were considered. The final reference list was generated on the basis of originality and relevance to the broad scope of this Review.

I) Introduction

Non-alcoholic fatty liver disease (NAFLD) is a spectrum of progressive disease ranging from isolated hepatic steatosis to non-alcoholic steatohepatitis (NASH), cirrhosis and hepatocellular carcinoma that has rapidly become the most common chronic liver disease worldwide.[1] NAFLD affects up to nearly 30% of the world´s adults, but it is much more frequent in individuals with metabolic disorders, affecting more than 90% of patients with severe obesity and up to ~70% of those with type 2 diabetes (T2D). [2,3] Rates of NASH, which is the key driver of liver fibrosis and cirrhosis, are also the highest in individuals with metabolic disorders (affecting up to ~40%).[4,5] The healthcare and socioeconomic costs of NAFLD and NASH are increasing globally [6]. It is known that cardiovascular disease is the predominant cause of mortality in people with NAFLD (up to nearly 40%), followed by extrahepatic cancers and liver-related complications.[7] NASH-related cirrhosis will become the major cause for liver transplantation in the next decade.[8,9] NAFLD reflects a prototypic multidisciplinary and multisystem disease [10], which cannot be managed by a single speciality group.[11,12] Public awareness of NAFLD and involvement of all stakeholders will facilitate managing NAFLD in the future.[9] In this review article, we discuss some of changes in pathophysiological concepts underpinning NASH over time, and how that has influenced NASH drug treatment development. We also highlight some of challenges and lessons that have been learnt from treating NAFLD, and we emphasise multidisciplinary treatment strategies for the future.

II) Timeline of the major targets in NAFLD: how pathophysiological concepts have changed over time and affected drug therapy

A causal link between liver fat and fibrosis development was proposed by the Austrian pathologist Rokitansky in the 19th century [13]. In 1938, the American pathologist Connor described the presence of fatty liver disease in patients with T2D.[14] In 1980, Dr. Ludwig and colleagues from the Mayo Clinic introduced the term “NASH” quoting “a hitherto unnamed liver disease that histologically mimics alcoholic hepatitis and that also may progress to cirrhosis”.[15] The link between liver fat and liver inflammation remained unclear and in 1998 Chris Day and Oliver James

proposed the “two hits hypothesis to NASH” suggesting that gut-derived signals (such as endotoxin) might drive liver inflammation mainly via induction of oxidative stress and proinflammatory cytokines.[16] Careful studies lead by Marchesini and colleagues demonstrated in 1999 that NAFLD is linked with greater insulin resistance and metabolic disorders.[17] One of us suggested in 2000 that proinflammatory cytokines might play a crucial role in the evolution of NASH[18] and in the following years, the pathophysiological complexity of the development and progression of NAFLD became more apparent. Various gut-derived signals along with dietary components or adipose-tissue derived signals were suggested to promote liver inflammation in NAFLD, allowing us to propose a “multiple hits hypothesis” for the disease.[19] Liver fibrosis is now recognised as the strongest risk factor for predicting prognosis of NAFLD.[20,21] NAFLD has been shown also to develop in lean subjects (although the pathophysiology of patients with so-called lean NAFLD is not well characterized[22]), and we now know that lean subjects with NAFLD have similar clinical outcomes compared to their overweight or obese counterparts with NAFLD.[22]

Current pathophysiological concepts

A key component of NAFLD pathophysiology is metabolic dysfunction. Whereas it was thought that the development of hepatic steatosis was a passive process caused by mal-/over-nutrition, it is now accepted that different factors, such as low-grade inflammation driven by cytokines, endoplasmic reticulum stress, autophagy or gut dysbiosis, may contribute to NAFLD development.[23,24] Further key mechanisms include an increased influx of lipids or free fatty acids (FFA) into the liver, increased de novo lipogenesis (DNL), and a decrease in fatty oxidation and lipid export from the liver.[25] Expanded visceral adipose tissue and its delivery of FFA from both visceral and subcutaneous adipose tissue is also pathophysiologically important. [26] Fructose overconsumption might cause not only hepatic steatosis, but also ER stress, NASH and hepatocellular carcinoma, at least in preclinical models.[27] Despite these facts, hepatic steatosis may occur without accompanying liver inflammation in ~80-90% of affected individuals and it is unclear why only 10-20% of individuals with isolated hepatic steatosis may progress towards NASH. Certain specific lipid species might cause liver inflammation (i.e., lipotoxicity), independent from additional microbial signals derived from the gut. Both free cholesterol and sphingolipids such as ceramides have been shown to promote liver inflammation.[28,29] Systemic and

hepatic insulin resistance, which is a key feature of NAFLD, is also driven by liver inflammation as inflammatory components, such as certain cytokines, may promote or exacerbate systemic/hepatic insulin resistance.[30] In this context, endotoxin may play a role as it has been detected within the liver in NAFLD.[31,32] In recent years, it has become evident that gut dysbiosis also occurs with NAFLD (especially with advanced fibrosis) and some animal and human evidence is now also accumulating to suggest that the gut-microbiome-liver axis might play a role in NAFLD development and especially in disease progression towards fibrosis and cirrhosis.[33-35] Many other ligands and receptors, such as bile acid species and related farnesoid X receptor (FXR) signalling, have also been demonstrated to reflect crucial pathways in NAFLD development.[36] It is beyond the scope of this article to describe and discuss the importance of genetic factors in NAFLD (including the genetic variants in *PNPLA3* rs738409 and *TM6SF2* rs58542926 or other NAFLD risk variants), and potential effects on disease progression and treatment.[32] A complex and pleiotropic array of pathophysiological components are contributing to NAFLD development, including genetic, dietary, metabolic, microbiome and immune-driven aspects.[25] Current potential treatment targets based on pathophysiological aspects are illustrated in **Figure 1**.

III) Past and current concepts in drug therapy

Soon after discovery of the importance of insulin resistance researchers tested metformin as a potential therapy for NAFLD. Initial studies showed no convincing benefit[37], and did not improve NAFLD histological features[38]. Various weight-loss inducing agents such as orlistat also showed no efficacy.[39] In 2010 the PIVENS (Pioglitazone vs. Vitamin E vs. Placebo for the Treatment of Nondiabetic Patients With NASH) trial compared the peroxisome proliferator activated receptor (PPAR)-gamma ligand pioglitazone (30 mg daily) with vitamin E (800 IU daily) or placebo in adults with NASH and without T2D.[40] The results of this randomized clinical trial formed the basis for treatment recommendations in the USA for specific non-cirrhotic NASH populations either using vitamin E or pioglitazone.[41] Whereas pioglitazone reflects a key drug interfering with insulin resistance, vitamin E-exerted mechanisms might be more diverse, including anti-inflammatory activities. Weight loss through

lifestyle modification was recognised as a highly successful strategy to improve liver histology in NAFLD.[42] The importance of weight loss was especially proven by large bariatric surgery studies where marked weight loss resulted in resolution of NASH in >80% of subjects and a progressive and substantial reduction of liver fibrosis in >70% of patients five years after bariatric surgery[43] establishing the important concept of NAFLD disease reversibility. Among patients with NASH and obesity, bariatric surgery procedures (compared with nonsurgical management) were also associated with a lower risk of incident major adverse liver outcomes and fatal/nonfatal cardiovascular events [44]. We showed that bariatric surgery-induced weight loss almost eliminated expression of proinflammatory cytokines both in the adipose tissue and liver.[45]

Mass spectrometry analyses in the European NAFLD registry revealed that certain mainly lipid-specific signatures exist across the whole NAFLD spectrum, and transition from F2 to F3 fibrosis stages reflects a turning point where oxidative stress and inflammatory responses become more dominant.[46] Metabolic targets in NAFLD include FXR with bile acids as major ligands, DNL or acetyl-CoA carboxylase (NB: drugs with a focus on insulin resistance/T2D pathways, including PPAR-alpha/delta and glucagon-like peptide 1 [GLP-1] receptor agonists are discussed later).

The most advanced phase 3 placebo-controlled trial in non-cirrhotic NASH used obeticholic acid (25 mg daily) as a modified bile acid ligand and selective FXR agonist.[47] Whereas this drug met its histologic endpoints with respect to improvement in liver fibrosis, this was not the case with resolution of NASH (although some individual features of NASH improved with the drug), and furthermore the limiting side effects of this drug that are likely affect future use of obeticholic acid may be pruritus and increased plasma low-density lipoprotein (LDL)-cholesterol concentrations.

Thyroid hormone receptor-beta (THR-β) mediates important metabolic and lipid pathways and phase 3 trials with resmetirom are currently ongoing. In a phase 2b placebo-controlled trial, treatment with resmetirom resulted in significant reduction in liver fat content (on magnetic resonance imaging) after 12 weeks and 36 weeks of

treatment in patients with biopsy-confirmed non-cirrhotic NASH [48]. The results of phase 3 placebo-controlled randomized trials with resmetirom (MAESTRO-NAFLD-1 and MAESTRO-NASH) have been announced and presented in abstract form, although full publication of the data are still awaited. Aramchol is a conjugate of a bile acid with a fatty acid suppressing hepatic steatosis by inhibiting stearoyl-CoA-desaturase 1 (SCD1), which reflects a rate-limiting step in fatty acid synthase. Phase 2b trial results were promising with higher rates of NASH resolution and also fibrosis improvement (but not significant in intention-to-treat analysis) [49] and this drug is now being tested in an ongoing phase 3 trial. The pleiotropic fibroblast growth factors FGF-19 and FGF-21 profoundly regulate energy homeostasis and lipid/glucose biology and affect sugar intake. To date, phase 2 clinical trials have so far been completed with this new class of drugs in NAFLD. Aldafermin, a FGF-19 analogue, failed to improve liver fibrosis in a phase 2b trial of patients with non-cirrhotic NASH and fibrosis, but positively affected various secondary endpoints[50], whereas pegozafermin, a glycoPEGylated FGF-21 analogue, beneficially affected liver fat content and circulating lipids[51] whereas efruxifermin, a long-acting Fc-FGF21 fusion protein, significantly reduced liver fat content (assessed by magnetic resonance imaging) in non-cirrhotic NASH.[52]

The dual chemokine receptor CCR2/CCR5 inhibition by cenicriviroc demonstrated anti-inflammatory and anti-fibrotic activities in various preclinical mouse models of liver diseases.[53] In the phase 2b CENTAUR trial involving patients with non-cirrhotic NASH and fibrosis, the initial improvement in liver fibrosis after one year of treatment was not sustained after two years of therapy.[54] In the AURORA phase-3 randomized placebo-controlled trial, treatment with cenicriviroc for 12 months lacked efficacy to treat liver fibrosis in patients with biopsy-confirmed non-cirrhotic NASH and liver fibrosis [55]. Consequently, further development of cenicriviroc has been stopped. Other anti-fibrotic drugs such the galectin-3 receptor agonist belapectin might reflect a drug for patients with advanced fibrosis and/or NASH-related cirrhosis but further confirmatory evidence of benefit in other studies is needed.[56] Anti-inflammatory strategies have so far not been followed (besides cenicriviroc) but might be considered in the future as: (i) systemic/liver inflammation, as assessed by C-reactive protein (CRP) or interleukin (IL)-1 receptor antagonist serum levels, may be relevant in NAFLD; and (ii) cardiovascular disease is the leading cause of death in

NAFLD. Furthermore, a recent collaborative analysis of three large randomized trials has suggested that among patients receiving statin therapy, low-grade inflammation assessed by plasma high-sensitivity CRP is a stronger predictor of future cardiovascular events than plasma LDL-cholesterol levels.[57] Lastly, several inhibitors are being investigated that target various enzymes (such as acetyl-CoA carboxylase [ACC], fatty acid synthase [FAS], stearoyl-CoA desaturase 1 [SCD1] or diacylglycerol acyltransferase 2 [DGAT2]) involved in the synthesis of triglycerides [58].

IV) Have trials designs and endpoints changed? Why and whether current efficacy/safety endpoints are suitable or not?

Starting from the first description of "NASH" by Ludwig and colleagues, liver histology has evolved as the cornerstone in diagnosing and staging this liver disease.[15] Kleiner and colleagues established in 2005 the histologic NAFLD activity score (NAS), including fibrosis staging which has been used in many clinical trials starting with the PIVENS trial.[59,40] Over the past 20 years, major advances have been made in our understanding of the biology of NAFLD, such as the recognition that the histological severity of liver fibrosis (fibrosis stage F≥2) is strongly associated with an increased risk of liver-related complications and all-cause mortality.[20,21] One of the most critical decisions in the NAFLD community has been the decision by the US Food and Drug Administration (FDA) in 2011 to define histological endpoints by either achieving resolution of NASH (defined as an inflammation score of zero or one and ballooning score of zero) without worsening of liver fibrosis or improvement in liver fibrosis by one stage or more, and without worsening of NASH, for conditional approval of new drugs for NASH.[40] This approach has several disadvantages and might be challenged in the future. First, liver biopsy reflects an invasive procedure with a very small but important risk of acute complications, e.g., major bleeding and moderate/severe pain [61]. Second, liver biopsy may introduce a possible sampling error as disease manifestation might differ within the liver. Third, we do not know currently whether liver inflammation is a stable process over days/weeks or might take place intermittently as observed in other extrahepatic inflammatory disorders. Fourth, there exist major issues regarding intra-

and inter-reader reliability in liver histology assessment, e.g., significant inter-reader variability does exist in assessing the presence and severity of hepatocyte ballooning, which is a key histological feature for the diagnosis of NASH [62,63]. It is likely that the use of machine learning and artificial intelligence approaches in the detection and interpretation of hepatocyte ballooning might provide new possibilities for a more accurate liver histology assessment [63]. Proposing as endpoints of clinical studies “NASH resolution and fibrosis improvement” places the bar very high, not least because stabilising (and not improving liver disease) may be sufficient to prevent liver endpoints. That said, whether stabilising and not improving liver disease would be sufficient to decrease risk of extrahepatic complications of NAFLD as a multisystem disease remains uncertain.

Safety issues have always been a major concern in drug approval to protect patients from severe and often rare adverse reactions, which are rarely recognised in phase 3 clinical trials. Here, drug-induced liver injury (DILI) is of special relevance as it is associated with acute liver failure and mortality. Identification of DILI might be challenging as it has to be separated from NAFLD and might therefore require a liver biopsy as part of investigating the differential diagnosis. For these reasons and especially also considering the “systemic nature” of NAFLD with mainly extrahepatic (cardiometabolic) clinical outcomes, biomarkers and non-invasive tests (NIT) as discussed below are urgently needed to improve treatment monitoring. The duration of clinical trials is also an important factor as liver fibrosis regression may take years to occur, especially when fibrosis is advanced in more severe disease.[64] This is particularly important for pharmacological trials that include patients with NASH-related cirrhosis. Furthermore, patient phenotypes can vary considerably, and we do not have “a one size fits all drug”. We are not faced with a lack of treatment options, but liver efficacy endpoints are not the only necessity when we are faced with NAFLD as a multisystem disease affecting extrahepatic organs and diseases.[10,65]

V) New outcome parameters awaited: non-invasive assessment of disease and consideration of NAFLD as a multisystem disease

In recent years, there have been many advances in the development of biomarkers and NITs for the diagnosis of NAFLD or NASH,[66] as well as in the consideration of NAFLD as a multisystem disease with important cardiometabolic implications.[10] For that reason, we believe that the effect of any new drug for NASH should also target cardiometabolic risk factors and risk of long term cardiovascular and metabolic events. Thus, in our opinion measurement of cardiometabolic risk factors should always be monitored in future clinical trials and included as end points. Emerging data suggest that a reduction in intra-abdominal fat depots, assessed by magnetic resonance imaging [MRI], is associated with histologic improvement in NASH.[67] Additionally, the presence of cardiometabolic comorbidities in clinical trials may also affect the therapeutic response rate of a NASH drug candidate, as some drugs may be more or less effective for specific NASH phenotypes. For that reason, stratification for major cardiometabolic comorbidities, such as T2D or obesity, is essential to further improve the reliability and reproducibility of NASH clinical trials.[68]

Biomarkers and NITs are emerging as reliable tools for assessing the likelihood of significant hepatic fibrosis, predicting risk of disease progression and adverse liver-related events, making management decisions, and to some degree, for assessing treatment response (although this last issue is poorly validated to date).[69-71] The results from the phase 3 REGENERATE trial (testing obeticholic acid) suggested that NITs may be useful alternatives to liver biopsy in assessing NASH patients' response to treatment [72]. However, we believe that further validation of biomarkers and NITs is certainly needed. The further validation of biomarkers and NITs will also facilitate the identification of patients with fibrotic NASH (i.e., patients with NASH with fibrosis stage F≥2, who are at greater risk of developing adverse liver-related outcomes) and their response to NASH drug candidates without the need for liver biopsies, as currently requested by the FDA in phase 3 clinical trials for conditional approval of new drugs for NASH.[73] In the next section, we briefly discuss the imaging-based methodologies that could be used to assess disease severity and longitudinal changes non-invasively in clinical practice. We do not discuss the use of

omics technologies because none of these biomarkers are validated for monitoring treatment responses in NASH.[74]

Imaging-based methodologies

Conventional liver ultrasonography and the controlled attenuation parameter (CAP), which is measured in conjunction with vibration-controlled transient elastography (using the Fibroscan® device), lack sufficient sensitivity for accurately quantifying or monitoring changes in hepatic fat content.[75]

MRI–proton density fat fraction (MRI-PDFF) has emerged as one of the most accurate and reproducible methods for hepatic fat quantification that is used in clinical research and tertiary care centers.[75] MRI-PDFF offers also promise as a non-invasive biomarker of treatment response in early-phase NASH clinical trials. In a meta-analysis of seven clinical trials that incorporated MRI-PDFF and examined histologic response following drug intervention in individuals with NASH, a ≥30% relative reduction in MRI-PDFF was found to be associated with a significant improvement in histologic NAS score and greater resolution of NASH.[76] Specifically, the MRI-PDFF responders were more likely to achieve NASH improvement than non-responders (by a factor of seven when treatment response was assessed by NAS reduction and by a factor of about five when assessed by NASH resolution).[76] Although further validation studies are required, these findings suggest the use of MRI-PDFF for non-invasive monitoring of treatment response in early-phase NASH trials, especially with drugs targeting metabolic pathways.[68]

Fibroscan® is the most widely used method to measure liver stiffness in clinical practice and can be used to accurately exclude significant fibrosis and cirrhosis. However, its accuracy for non-invasive monitoring of treatment responses in NASH remains to be further validated.[66,70,76] Magnetic resonance elastography (MRE) is now considered the best method for non-invasively quantifying hepatic fibrosis in NAFLD.[77-80] A recent head-to-head comparison between various imaging-based elastography methods with liver histology found that MRE was superior to other imaging-based elastography methods for detection of any degree of fibrosis.[75] Liver stiffness, measured by MRE, also predicts the future risk of adverse liver-related and cardiovascular events.[81,82] However, further validation studies are needed to clarify

whether MRE-assessed liver stiffness could replace liver biopsy for the longitudinal assessment of treatment response in NASH clinical trials.[75,83] An additional issue that needs to be addressed is that few centres currently have access to the hardware that is needed to make the necessary measurements.

Techniques combining simple clinical parameters with imaging-based elastography methodologies that may be predictive of liver-related clinical outcomes are now emerging. The Fibroscan-AST (FAST) score (combining liver stiffness and CAP measured by Fibroscan® with serum aspartate aminotransferase levels), the MAST score (combining MRI-PDFF with serum aspartate aminotransferase levels) and the MEFIB index (combining MRE with fibrosis-4 [FIB-4] index) can be efficiently used for the non-invasive identification of fibrotic NASH.[84-86] A recent head-to-head comparison between these three combined scores reported that the MEFIB index was better than MAST and FAST scores for the non-invasive diagnosis of fibrotic NASH.[87] In addition, the MEFIB index and the MAST score accurately predicted liver-related mortality and events in this patient group.[88,89]

Other emerging imaging-based modalities of interest include multiparametric liver MRI that could be a good diagnostic option for evaluating disease severity in NAFLD. This imaging methodology (that uses a *LiverMultiScan*® software with sequences for corrected T1 mapping [cT1], MRI-PDFF and T2* mapping) accurately identified moderate-to-severe steatosis, NASH and advanced fibrosis.[90-94] Emerging data showed that multiparametric liver MRI data were correlated with histological features of NASH and also accurately detected longitudinal changes in hepatic inflammation and fibrosis.[95] Studies conducted in UK found that multiparametric liver MRI was cost effective for NAFLD risk stratification, and combined with Fibroscan® had the lowest cost per correct diagnosis.[96,97]

Liver biopsies are not feasible for monitoring responses to pharmacological treatments in clinical practice. One of the highest priorities is the validation of imaging-based methodologies (alone or in combination with simple clinical parameters or with more sophisticated biomarkers) to assess disease severity and monitor treatment responses. It is crucial to define the exact decrease in liver stiffness by MRE or other imaging-based methodologies that is associated with a

histologic improvement in liver fibrosis of at least one stage or resolution of NASH or lower risk of liver-related and cardiometabolic events. These data are needed to ensure endorsement by the FDA and other regulatory authorities of these imaging-based methodologies as alternatives to histological endpoints in future phase 3 NASH therapeutic trials.[68] Validation of existing NITs as outcome parameters of treatment response will accelerate the development and approval of new drugs for NASH and allow monitoring of the treatment response in clinical practice. Since there has been a substantial increase in the number of studies applying artificial intelligence (AI) in medical image analysis for the diagnosis of liver diseases over the past decade,[98] it is also reasonable to hypothesize that AI approaches using imaging-based methodologies will become soon available to develop highly accurate models for predicting disease severity and for non-invasive monitoring of treatment responses in NASH.[98,99] **Figure 2** summarizes the beneficial liver-related and cardiometabolic effects of an "ideal" drug candidate for NASH.

VI) Potential lessons from selected drug development stories: why some specific glucose-lowering medications could form the basis of future drug development

In the last 15 years, promising results in the treatment of NASH with two specific treatment options for T2D have led to further evolution and targeting of these therapeutic modalities in the treatment of NASH. This is an evolving area and within the limits of this review, we have chosen to focus on peroxisome proliferator-activated receptors (PPARs) and incretin receptor and glucagon receptor agonists, which we consider could have a marked effect on the treatment of NAFLD as a multisystem disease over the next decade.

Peroxisome proliferator-activated receptors

PPARs are ligand-activated nuclear transcription factors known to regulate glucose and lipid metabolism, inflammation, endothelial function and fibrosis [100].[101] PPARs are a nuclear hormone receptor superfamily comprising: PPAR-alpha, PPAR-gamma and PPAR-beta/delta (thereby referred to as delta). Activated PPARs are capable of transcriptional repression through DNA-independent protein-protein interactions with

other transcription factors, such as nuclear factor kappa B signal activators and transducers of transcription.[102] PPAR-alpha activation reduces plasma triglyceride concentrations and is involved in regulation of energy homeostasis. PPAR-gamma activation improves insulin sensitivity and enhances glucose metabolism, whereas PPAR-delta activation enhances fatty acids metabolism.[101]

PPARs were identified in 1990[101] and the first of the PPAR-gamma agonist thiazolidinedione class of drugs that had powerful glucose-lowering effects was troglitazone, which was launched in the USA in 1997.[103] Subsequently, rosiglitazone and pioglitazone were licensed in the USA in 1999. Pioglitazone has many potential benefits that extend beyond its glucose-lowering effects to the vasculature and the liver, and in 2019 De Fronzo and colleagues summarised the beneficial effects of pioglitazone on the vasculature in T2D concluding that pioglitazone has become the "forgotten, clinically effective, cost effective treatment for patients with T2D".[104] New data in the last decade showed that pioglitazone is also an effective treatment for non-cirrhotic NASH in patients both with, and without T2D (mainly in terms of histological resolution of NASH).[105-107]

Recent data extend the key role of PPARs to liver diseases occurring with vascular dysfunction, including NAFLD.[108] PPAR-gamma is expressed in many tissues but its splicing isoform (PPAR-gamma2) is expressed solely in adipocytes and PPAR-gamma activation increases the production of adiponectin from adipocytes[109] that is an adipokine protein with insulin-sensitizing and anti-inflammatory effects.

As mentioned earlier, NAFLD is a multisystem disease that not only affects the liver but also increases risk of developing cardiovascular disease and other extrahepatic diseases.[10,65] PPAR-gamma activation has beneficial effects on lipid metabolism, insulin sensitivity, oxidative stress, endothelial function, inflammatory pathways and coagulation and effects on all these processes could have favourable consequences for the vasculature in patients with NAFLD. Although the benefit of agonism of other PPARs (beyond PPAR-gamma) on liver disease is unproven, the recent results from a phase 2b randomized, placebo-controlled trial testing the effect of lanifibranor suggested that this pan-PPAR agonist benefits liver disease.[110] In this clinical trial, involving 247 patients with non-cirrhotic NASH, the authors found that the

percentage of patients who had a decrease of at least 2 points in the SAF-A score (i.e. the activity part of the Steatosis, Activity, Fibrosis [SAF] scoring system that incorporates scores for hepatocyte ballooning and inflammation) without worsening of fibrosis was higher with the 1200-mg dose of lanifibranor than with placebo. Another small phase 2 randomized, placebo-controlled trial testing the effect of saroglitazar suggested that this dual PPAR-alpha/gamma agonist improved liver fat content (on MRI-PDFF), insulin resistance, and atherogenic dyslipidaemia in patients with NAFLD [111].

PPAR-alpha agonism modulates fatty acid oxidation in the liver[112] and effectively reduces plasma triglyceride concentrations associated with the metabolic syndrome[113] and the altherogenic lipoprotein phenotype[114], which are very common conditions in NAFLD. However, the failure of elafibranor (a PPAR-alpha and delta agonist) to show benefit in patients with non-cirrhotic NASH in a phase 3 randomized trial, and the current uncertainties surrounding a possible benefit from elafibranor,[115] may provide insight into the fact that the benefit of lanifibranor might derive mainly from its PPAR-gamma agonist effects. However, further research is needed to better clarify this issue.

GLP-1, glucose-dependent insulinotropic peptide (GIP) and glucagon receptor agonists

We have recently discussed in detail in this Journal, the potential benefits of GLP-1 and GIP receptor agonism in NAFLD.[116] In particular, a number of recent phase 2 randomized controlled trials showed that GLP-1 receptor agonists (especially subcutaneous liraglutide and semaglutide, which have demostrated also to have beneficial effects on histological NASH resolution without worsening of liver fibrosis) or tirzepatide, a dual GLP-1/GIP co-agonist, are two promising treatment options for patients with NAFLD or non-cirrhotic NASH that warrant further investigation [107,116]. In a phase-2 placebo-controlled trial of patients with NASH-related cirrhosis, treatment with semaglutide 2·4 mg once weekly for 48 weeks did not improve fibrosis or achievement of NASH resolution [117]. In this section, we extend the discussion to include combination incretin receptor effects with glucagon receptor agonism in the treatment of NAFLD as a multisystem disease. Specifically, dual or even triple receptor agonism with GLP-1/GIP receptor agonism combined with

glucagon receptor agonism may provide extra therapeutic benefits, and the effects of glucagon receptor agonism combined with GLP-1 and GIP receptor agonisms in NAFLD are schematically illustrated in **Figure 3.** This figure illustrates the utility of these agents in the treatment of NAFLD as a multisystem disease. In particular, the figure highlights the potential benefits of incretin receptor agonists and combination therapy with glucagon receptor agonists on processes relevant to both liver disease and the extrahepatic consequences of liver disease.

Dual GLP-1/glucagon receptor co-agonists are currently being studied, such as BI456906 in a phase 2b placebo-controlled trial (NCT04771273), and efinopegdutide in a phase 2a active-comparator-controlled trial (NCT04944992). Triple receptor agonists that also include GIP receptor signalling agonists are also showing promise and need further testing. HM15211 is a triple GLP-1/GIP/glucagon receptor agonist that showed promising results in a phase 1b/2a trial in obese individuals with NAFLD, with a maximal ~90% reduction of liver fat content at 12 weeks with the highest dose of drug.[118] A phase 2b placebo-controlled trial is now ongoing to evaluate the efficacy of HM15211 in NASH (NCT04505436). Another potential therapeutic effect that needs testing is to enhance GLP-1 receptor agonism and increase GLP-1 signalling to both increase insulin secretion and weight loss effects.

In contrast to incretins, glucagon acts predominantly and directly on the liver, via the glucagon receptor. The glucagon receptor is a 62kDa protein that is a member of the G-protein coupled family of receptors. Stimulation of the glucagon receptor results in activation of a common signal transduction pathway that includes adenylate cyclase and phospholipase C, thereby resulting in increased concentrations of intracellular calcium and cAMP as second messenger signalling molecules. Importantly, and relevant to NAFLD as a multisystem disease, glucagon receptors are expressed not only in the liver, but also in the kidney, adipose tissue, heart, gastrointestinal tract and also adrenal glands, cerebral cortex and pancreas. [119]

Over 25 years ago, it was shown that a missense mutation in the glucagon receptor was associated with the presence of T2D,[120] suggesting that normal functioning of this catabolic hormone was important for maintaining normoglycaemia. Recently, a

better awareness of other effects of glucagon receptor agonism has been promoted by newer drug classes, such as dipeptidyl peptidase-4 inhibitors and GLP-1 receptor agonists that lower blood glucose levels and yet decrease glucagon secretion. This action and the role of glucagon as a catabolic hormone that promotes ketogenesis[121] has been responsible for increasing interest in the role of glucagon receptor agonism in NAFLD. Unlike GIP and GLP-1 that do not act directly on the liver, glucagon receptors are highly expressed in the liver and glucagon agonism has marked effects on metabolism in the liver and extra-hepatic tissues. The biological actions of glucagon are very relevant both to liver disease in NAFLD and to the effects of NAFLD as a multisystem disease.[10,65] The effects of glucagon extend to regulation of energy intake, stimulation of brown fat thermogenesis, inhibition of gastric motility, modulation of lipid metabolism through activation of lipolysis and inhibition of lipid synthesis, improvement of cardiac output, and increased heart rate and renal glomerular filtration (summarised in [119]). Besides promoting hepatic gluconeogenesis, glucagon also increases bile acid production, lipid oxidation and ketone body production, and decreases lipid synthesis. Glucagon's positive chronotropic and ionotropic effects on the heart have been known about since the 1960s and in humans with heart disease glucagon increases heart rate, cardiac output, cardiac index, stroke power index and left ventricular pressure.[122]

Pemvidutide (ALT-801) is a drug that targets both the glucagon receptor and the GLP-1 receptor and is now being tested in NAFLD. Pemvidutide may also prove useful for the treatment of obesity and T2D. This synthetic peptide is administered subcutaneously and is an analogue of oxyntomodulin. The peptide targets both the GLP-1 and glucagon receptors. In September 2022, Altimmune announced significant reductions in liver fat content and body weight in a 12-week phase 1b trial of pemvidutide in patients with NAFLD.[123] Mean weight loss (placebo-adjusted) was only 4.7% in NAFLD patients without diabetes who received 1.8 mg dose at 12 weeks of treatment.[123] Data presented in abstract form at the American Heart Association meeting in November 2022, reported some beneficial effects of pemvidutide on plasma lipids.[124] These data showed that pemvidutide achieved significant dose-dependent reductions from baseline across multiple bioactive lipid classes, including pro-atherogenic lysophosphatidylcholines (a major oxidized LDL component), and lipotoxic sphingolipids, including sphingomyelins and ceramides.

Thus, in summary it is plausible that combination therapy with a peptide that targets the glucagon receptor and the GLP-1 and GIP receptors could prove very effective in not only treating liver disease, but also targeting the extrahepatic complications of NAFLD as multisystem disease.

VII) Conclusions

Despite the high global prevalence and the detrimental effects of NAFLD on life-expectancy and risk of hepatic and extrahepatic complications, there is currently no licensed pharmacotherapy available for this burdensome metabolic liver disease. We are not faced with a lack of treatment options, but efficacy end points might not always be suitable for treatment of this multisystem disease affecting multiple extrahepatic organs and diseases. Liver biopsy is not feasible for monitoring treatment responses in clinical practice. Validation of existing NITs and imaging modalities as outcome parameters of treatment response will accelerate the development and approval of new drugs for NASH and allow monitoring of treatment responses in clinical practice. To date, it remains to be shown in phase 3 trials whether the anticipated benefit attributable to lanifibranor (a pan-PPAR agonist) in patients with non-cirrhotic NASH, extends beyond the benefit expected from PPAR-gamma agonism alone. In addition, larger clinical trials with a long treatment duration are needed to investigate whether these and other drugs for NASH can improve adverse liver-related outcomes in patients with NASH and compensated cirrhosis. The challenge for the future is also to know for certain whether combination effects of GLP-1, GIP and glucagon receptor agonism benefits NAFLD as a multisystem disease that has important consequences beyond the liver.

Acknowledgements: HT is supported by the excellence initiative VASCage (Centre for Promoting Vascular Health in the Ageing Community), an R&D K-Centre (COMET program - Competence Centers for Excellent Technologies) funded by the Austrian Ministry for Transport, Innovation and Technology, the Austrian Ministry for Digital and Economic Affairs and the federal states Tyrol, Salzburg and Vienna. CDB is supported in part by the Southampton National Institute for Health and Care

Research UK, Biomedical Research Centre NIHR *grant code,* NIHR203319. GT is supported in part by grants from the University School of Medicine of Verona, Verona, Italy.

Figure Legends

Figure 1: Current treatment targets based on pathophysiological concepts in NASH.

The underlying pathophysiology is complex and involves various pathways including metabolic, inflammatory, fibrosis, microbiome and immune concepts. 1: Lipid-specific signatures exist across the whole NAFLD spectrum and lipotoxicity plays a role also in inducing “sterile” liver inflammation. Several drugs are currently tested in phase 2 and 3 studies targeting metabolic/lipid pathways, e.g. FXR agonists, PPARα/δ agonists, aramchol, THR-β agonists, ACC inhibitors or FGF19/21 analogues. 2: Insulin resistance (especially hepatic insulin resistance) is a key feature of NAFLD and drugs used for the treatment of type 2 diabetes are currently intensively studied in NAFLD, e.g. GLP-1 receptor agonists, dual GLP-1/GIP receptor co-agonists, glucagon receptor agonists, SGLT-2 inhibitors. 3: Inflammation is a key driving force of NASH, either driven by sterile processes or potentially microbial products and mainly responsible for fibrosis development. Although cenicriviroc (a CCR2/5 inhibitor) has failed in a phase 3 trial improving fibrosis, targeting inflammation by specific drugs remains a key strategy in targeting NASH. NLRP3 inflammasome inhibitors have so far been tested in preclinical NAFLD models. A side-chain shorted derivate of ursodeoxycholic acid i.e. norursodeoxycholic acid also acts anti-inflammatory and has been tested in phase 2 studies. 4: Liver fibrosis is the key prognostic factor for NAFLD and therefore targeting fibrosis and its potential reversibility remains a key aspect in drug development. Galectin-3 receptor agonists are another group of drugs being currently tested. 5: Extrahepatic tissues are of crucial importance in this multisystem disease and both extrahepatic tissues and the liver are critically affected by NASH treatment (e.g., the adipose tissue for PPAR-gamma agonists; weight loss from GLP-1 RA and probably GIP agonists; the brain for GLP-1 RA, and the kidneys for SGLT-2 inhibitors). 6: Gut microbiome is altered especially in more advanced NAFLD and targeting the microbiome might evolve in the future as an attractive treatment strategy. Many drugs mentioned in this Figure are pleiotropic and exert in addition anti-inflammatory and anti-fibrotic effects.

Abbreviations: ACC, acetyl-CoA carboxylase; DNL, de novo lipogenesis; GIP, glucose-dependent insulinotropic polypeptide; GLP-1RA, glucagon-like peptide 1 receptor agonists; JNK, c-jun N-terminal kinase; NLRP3, NLR family pyrin domain

containing 3; PPAR, peroxisome proliferator-activated receptor; ROS, reactive oxidative stress; SGLT-2, sodium-glucose linked transporter 2.

Figure 2: Effects of an ideal new drug for NASH therapy.
Since NASH is just one facet of a multisystem disease that confers increased morbidity and mortality on patients affected and where the leading cause of mortality is cardiovascular disease, an "ideal" drug candidate for the treatment of NASH should exert not only hepatoprotective effects (with improvement in histological features of NASH) but also beneficial effects on cardiovascular system (with reduction of cardiometabolic risk factors). These beneficial effects on the liver, heart and vasculature should translate into a long-term risk reduction of major liver-related and cardiovascular events in patients with NASH. Liver biopsy assessment is not easy to perform especially for the evaluation of response treatment in clinical practice. Validation of existing imaging-based methods (alone or in combination with other non-invasive biomarkers) as outcome measures of treatment response will accelerate the development and approval of new drugs for NASH and allow monitoring of the treatment response also in clinical practice.
Abbreviations: NASH, non-alcoholic steatohepatitis; MR, magnetic resonance; MRI-PDFF, magnetic resonance imaging–proton density fat fraction.

Figure 3: Treatment of NAFLD as a multisystem disease: Potential benefits of incretin receptor agonists and combination therapy with glucagon receptor agonists on processes relevant to both liver disease and the extrahepatic consequences of liver disease.
The effects of glucagon receptor agonism extend to regulation of energy intake, stimulation of brown fat thermogenesis, modulation of lipid metabolism through activation of lipolysis and inhibition of lipid synthesis. Additionally, glucagon receptor agonism improves cardiac output and renal glomerular filtration but causes increased heart rate. Besides promoting hepatic gluconeogenesis, glucagon also increases bile acid production, lipid oxidation and ketone body production, and decreases lipid synthesis. Glucagon's positive chronotropic and ionotropic effects on the heart have been known for many years and notably in humans with heart disease glucagon increases heart rate, cardiac output, cardiac index, stroke power index and left ventricular pressure. GLP-1 receptor agonists have proven efficacy to benefit T2D,

CVD and CKD. GLP-1 receptor agonists are effective in the brain and decrease appetite and induce satiety decreasing dietary calorie intake. These effects can facilitate weight loss and weight loss also benefits NAFLD as well as type 2 diabetes and CVD risk factors. Dual GLP-1 and GIP receptor co-agonists maybe more effective at promoting weight loss than GLP-1 receptor agonists alone. Thus, dual GLP-1 and GIP receptor co-agonists may prove to be very effective treatments for NAFLD or NASH as well as the extrahepatic complications of NAFLD as a multisystem disease.

Abbreviations: T2D, type 2 diabetes; GIP, glucose-dependent insulinotropic polypeptide; GLP-1, glucagon-like peptide-1; CVD, cardiovascular disease; CKD, chronic kidney disease.

References

1. Henry L, Paik J, Younossi ZM. Review article: the epidemiologic burden of non-alcoholic fatty liver disease across the world. *Aliment Pharmacol Ther* 2022; **56**(6): 942-56.
2. Younossi ZM, Golabi P, Paik JM, Henry A, Van Dongen C, Henry L. The global epidemiology of nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH): a systematic review. *Hepatology* 2023; **77**(4): 1335-47.
3. Tilg H, Moschen AR, Roden M. NAFLD and diabetes mellitus. *Nat Rev Gastroenterol Hepatol* 2017; **14**(1): 32-42.
4. Younossi ZM, Golabi P, de Avila L, et al. The global epidemiology of NAFLD and NASH in patients with type 2 diabetes: A systematic review and meta-analysis. *J Hepatol* 2019; **71**(4): 793-801.
5. Targher G, Corey KE, Byrne CD, Roden M. The complex link between NAFLD and type 2 diabetes mellitus - mechanisms and treatments. *Nat Rev Gastroenterol Hepatol* 2021; **18**(9): 599-612.
6. Schattenberg JM, Lazarus JV, Newsome PN, et al. Disease burden and economic impact of diagnosed non-alcoholic steatohepatitis in five European countries in 2018: A cost-of-illness analysis. *Liver Int* 2021; **41**(6): 1227-42.
7. Mantovani A, Csermely A, Petracca G, et al. Non-alcoholic fatty liver disease and risk of fatal and non-fatal cardiovascular events: an updated systematic review and meta-analysis. *Lancet Gastroenterol Hepatol* 2021; **6**(11): 903-13.
8. Noureddin M, Vipani A, Bresee C, et al. NASH Leading Cause of Liver Transplant in Women: Updated Analysis of Indications For Liver Transplant and Ethnic and Gender Variances. *Am J Gastroenterol* 2018; **113**(11): 1649-59.
9. Karlsen TH, Sheron N, Zelber-Sagi S, et al. The EASL-Lancet Liver Commission: protecting the next generation of Europeans against liver disease complications and premature mortality. *Lancet* 2022; **399**(10319): 61-116.
10. Targher G, Tilg H, Byrne CD. Non-alcoholic fatty liver disease: a multisystem disease requiring a multidisciplinary and holistic approach. *Lancet Gastroenterol Hepatol* 2021; **6**(7): 578-88.
11. Diehl AM, Farpour-Lambert NJ, Zhao L, Tilg H. Why we need to curb the emerging worldwide epidemic of nonalcoholic fatty liver disease. *Nat Metab* 2019; **1**(11): 1027-9.
12. Lazarus JV, Mark HE, Anstee QM, et al. Advancing the global public health agenda for NAFLD: a consensus statement. *Nat Rev Gastroenterol Hepatol* 2022; **19**(1): 60-78.
13. Ayonrinde OT. Historical narrative from fatty liver in the nineteenth century to contemporary NAFLD - Reconciling the present with the past. *JHEP Rep* 2021; **3**(3): 100261.
14. Connor CL. Fatty infiltration of the liver and the development of cirrhosis in diabetes and chronic alcoholism. *Am J Pathol* 1938; **14**(3): 347-64 9.
15. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin Proc* 1980; **55**(7): 434-8.
16. Day CP, James OF. Steatohepatitis: a tale of two "hits"? *Gastroenterology* 1998; **114**(4): 842-5.
17. Marchesini G, Brizi M, Morselli-Labate AM, et al. Association of nonalcoholic fatty liver disease with insulin resistance. *Am J Med* 1999; **107**(5): 450-5.
18. Tilg H, Diehl AM. Cytokines in alcoholic and nonalcoholic steatohepatitis. *N Engl J Med* 2000; **343**(20): 1467-76.
19. Tilg H, Moschen AR. Evolution of inflammation in nonalcoholic fatty liver disease: the multiple parallel hits hypothesis. *Hepatology* 2010; **52**(5): 1836-46.
20. Angulo P, Kleiner DE, Dam-Larsen S, et al. Liver Fibrosis, but No Other Histologic Features, Is Associated With Long-term Outcomes of Patients With Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2015; **149**(2): 389-97 e10.

21. Ekstedt M, Hagstrom H, Nasr P, et al. Fibrosis stage is the strongest predictor for disease-specific mortality in NAFLD after up to 33 years of follow-up. *Hepatology* 2015; **61**(5): 1547-54.
22. Younes R, Govaere O, Petta S, et al. Caucasian lean subjects with non-alcoholic fatty liver disease share long-term prognosis of non-lean: time for reappraisal of BMI-driven approach? *Gut* 2022; **71**(2): 382-90.
23. Li Z, Yang S, Lin H, et al. Probiotics and antibodies to TNF inhibit inflammatory activity and improve nonalcoholic fatty liver disease. *Hepatology* 2003; **37**(2): 343-50.
24. Ozcan U, Cao Q, Yilmaz E, et al. Endoplasmic reticulum stress links obesity, insulin action, and type 2 diabetes. *Science* 2004; **306**(5695): 457-61.
25. Tilg H, Adolph TE, Dudek M, Knolle P. Non-alcoholic fatty liver disease: the interplay between metabolism, microbes and immunity. *Nat Metab* 2021; **3**(12): 1596-607.
26. Jacobs K, Brouha S, Bettencourt R, Barrett-Connor E, Sirlin C, Loomba R. Association of Nonalcoholic Fatty Liver Disease With Visceral Adiposity but Not Coronary Artery Calcification in the Elderly. *Clin Gastroenterol Hepatol* 2016; **14**(9): 1337-44 e3.
27. Todoric J, Di Caro G, Reibe S, et al. Fructose stimulated de novo lipogenesis is promoted by inflammation. *Nat Metab* 2020; **2**(10): 1034-45.
28. Ioannou GN. The Role of Cholesterol in the Pathogenesis of NASH. *Trends Endocrinol Metab* 2016; **27**(2): 84-95.
29. Alsamman S, Christenson SA, Yu A, et al. Targeting acid ceramidase inhibits YAP/TAZ signaling to reduce fibrosis in mice. *Sci Transl Med* 2020; **12**(557).
30. Saltiel AR, Olefsky JM. Inflammatory mechanisms linking obesity and metabolic disease. *J Clin Invest* 2017; **127**(1): 1-4.
31. Carpino G, Del Ben M, Pastori D, et al. Increased Liver Localization of Lipopolysaccharides in Human and Experimental NAFLD. *Hepatology* 2020; **72**(2): 470-85.
32. Sookoian S, Pirola CJ, Valenti L, Davidson NO. Genetic Pathways in Nonalcoholic Fatty Liver Disease: Insights From Systems Biology. *Hepatology* 2020; **72**(1): 330-46.
33. Sharpton SR, Schnabl B, Knight R, Loomba R. Current Concepts, Opportunities, and Challenges of Gut Microbiome-Based Personalized Medicine in Nonalcoholic Fatty Liver Disease. *Cell Metab* 2021; **33**(1): 21-32.
34. Leung H, Long X, Ni Y, et al. Risk assessment with gut microbiome and metabolite markers in NAFLD development. *Sci Transl Med* 2022; **14**(648): eabk0855.
35. Tilg H, Adolph TE, Trauner M. Gut-liver axis: Pathophysiological concepts and clinical implications. *Cell Metab* 2022; **34**(11): 1700-18.
36. Radun R, Trauner M. Role of FXR in Bile Acid and Metabolic Homeostasis in NASH: Pathogenetic Concepts and Therapeutic Opportunities. *Semin Liver Dis* 2021; **41**(4): 461-75.
37. Bugianesi E, Gentilcore E, Manini R, et al. A randomized controlled trial of metformin versus vitamin E or prescriptive diet in nonalcoholic fatty liver disease. *Am J Gastroenterol* 2005; **100**(5): 1082-90.
38. Haukeland JW, Konopski Z, Eggesbo HB, et al. Metformin in patients with non-alcoholic fatty liver disease: a randomized, controlled trial. *Scand J Gastroenterol* 2009; **44**(7): 853-60.
39. Wang H, Wang L, Cheng Y, Xia Z, Liao Y, Cao J. Efficacy of orlistat in non-alcoholic fatty liver disease: A systematic review and meta-analysis. *Biomed Rep* 2018; **9**(1): 90-6.
40. Sanyal AJ, Chalassani N, Kowdley KV, et al. Pioglitazone, vitamin E, or placebo for nonalcoholic steatohepatitis. *New Engl J Med* 2010; **362**(18): 1675-85.
41. Chalasani N, Younossi Z, Lavine JE, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology* 2018; **67**(1): 328-57.

42. Vilar-Gomez E, Martinez-Perez Y, Calzadilla-Bertot L, et al. Weight Loss Through Lifestyle Modification Significantly Reduces Features of Nonalcoholic Steatohepatitis. *Gastroenterology* 2015; **149**(2): 367-78 e5; quiz e14-5.
43. Lassailly G, Caiazzo R, Ntandja-Wandji LC, et al. Bariatric Surgery Provides Long-term Resolution of Nonalcoholic Steatohepatitis and Regression of Fibrosis. *Gastroenterology* 2020; **159**(4): 1290-301 e5.
44. Aminian A, Al-Kurd A, Wilson R, et al. Association of Bariatric Surgery With Major Adverse Liver and Cardiovascular Outcomes in Patients With Biopsy-Proven Nonalcoholic Steatohepatitis. *JAMA* 2021; **326**(20): 2031-42.
45. Moschen AR, Molnar C, Enrich B, Geiger S, Ebenbichler CF, Tilg H. Adipose and liver expression of interleukin (IL)-1 family members in morbid obesity and effects of weight loss. *Mol Med* 2011; **17**(7-8): 840-5.
46. McGlinchey AJ, Govaere O, Geng D, et al. Metabolic signatures across the full spectrum of non-alcoholic fatty liver disease. *JHEP Rep* 2022; **4**(5): 100477.
47. Younossi ZM, Ratziu V, Loomba R, et al. Obeticholic acid for the treatment of non-alcoholic steatohepatitis: interim analysis from a multicentre, randomised, placebo-controlled phase 3 trial. *Lancet* 2019; **394**(10215): 2184-96.
48. Harrison SA, Bashir MR, Guy CD, et al. Resmetirom (MGL-3196) for the treatment of non-alcoholic steatohepatitis: a multicentre, randomised, double-blind, placebo-controlled, phase 2 trial. *Lancet* 2019; **394**(10213): 2012-24.
49. Ratziu V, de Guevara L, Safadi R, et al. Aramchol in patients with nonalcoholic steatohepatitis: a randomized, double-blind, placebo-controlled phase 2b trial. *Nat Med* 2021; **27**(10): 1825-35.
50. Harrison SA, Abdelmalek MF, Neff G, et al. Aldafermin in patients with non-alcoholic steatohepatitis (ALPINE 2/3): a randomised, double-blind, placebo-controlled, phase 2b trial. *Lancet Gastroenterol Hepatol* 2022; **7**(7): 603-16.
51. Loomba R, Lawitz EJ, Frias JP, et al. Safety, pharmacokinetics, and pharmacodynamics of pegozafermin in patients with non-alcoholic steatohepatitis: a randomised, double-blind, placebo-controlled, phase 1b/2a multiple-ascending-dose study. *Lancet Gastroenterol Hepatol* 2023; **8**(2): 120-32.
52. Harrison SA, Ruane PJ, Freilich BL, et al. Efruxifermin in non-alcoholic steatohepatitis: a randomized, double-blind, placebo-controlled, phase 2a trial. *Nat Med* 2021; **27**(7): 1262-71.
53. Krenkel O, Puengel T, Govaere O, et al. Therapeutic inhibition of inflammatory monocyte recruitment reduces steatohepatitis and liver fibrosis. *Hepatology* 2018; **67**(4): 1270-83.
54. Ratziu V, Sanyal A, Harrison SA, et al. Cenicriviroc Treatment for Adults With Nonalcoholic Steatohepatitis and Fibrosis: Final Analysis of the Phase 2b CENTAUR Study. *Hepatology* 2020; **72**(3): 892-905.
55. Anstee QM, Neuschwander-Tetri BA, Wai-Sun Wong V, et al. Cenicriviroc Lacked Efficacy to Treat Liver Fibrosis in Nonalcoholic Steatohepatitis: AURORA Phase III Randomized Study. *Clin Gastroenterol Hepatol* 2023.
56. Chalasani N, Abdelmalek MF, Garcia-Tsao G, et al. Effects of Belapectin, an Inhibitor of Galectin-3, in Patients With Nonalcoholic Steatohepatitis With Cirrhosis and Portal Hypertension. *Gastroenterology* 2020; **158**(5): 1334-45 e5.
57. Ridker PM, Bhatt DL, Pradhan AD, et al. Inflammation and cholesterol as predictors of cardiovascular events among patients receiving statin therapy: a collaborative analysis of three randomised trials. *Lancet* 2023.
58. Ferguson D, Finck BN. Emerging therapeutic approaches for the treatment of NAFLD and type 2 diabetes mellitus. *Nat Rev Endocrinol* 2021; **17**(8): 484-95.

59. Kleiner DE, Brunt EM, Van Natta M, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. *Hepatology* 2005; **41**(6): 1313-21.
60. Sanyal AJ, Brunt EM, Kleiner DE, et al. Endpoints and clinical trial design for nonalcoholic steatohepatitis. *Hepatology* 2011; **54**(1): 344-53.
61. Thomaides-Brears HB, Alkhouri N, Allende D, et al. Incidence of Complications from Percutaneous Biopsy in Chronic Liver Disease: A Systematic Review and Meta-Analysis. *Dig Dis Sci* 2022; **67**(7): 3366-94.
62. Davison BA, Harrison SA, Cotter G, et al. Suboptimal reliability of liver biopsy evaluation has implications for randomized clinical trials. *J Hepatol* 2020; **73**(6): 1322-32.
63. Li YY, Zheng TL, Xiao SY, et al. Hepatocytic ballooning in non-alcoholic steatohepatitis: Dilemmas and future directions. *Liver Int* 2023; **43**(6): 1170-82.
64. Sanyal AJ, Anstee QM, Trauner M, et al. Cirrhosis regression is associated with improved clinical outcomes in patients with nonalcoholic steatohepatitis. *Hepatology* 2022; **75**(5): 1235-46.
65. Byrne CD, Targher G. NAFLD: A multisystem disease. *J Hepatol* 2015; **62**(1S): S47-S64.
66. Anstee QM, Castera L, Loomba R. Impact of non-invasive biomarkers on hepatology practice: Past, present and future. *J Hepatol* 2022; **76**(6): 1362-78.
67. Shen W, Middleton MS, Cunha GM, et al. Changes in abdominal adipose tissue depots assessed by MRI correlate with hepatic histologic improvement in non-alcoholic steatohepatitis. *J Hepatol* 2023; **78**(2): 238-46.
68. Harrison SA, Allen AM, Dubourg J, Noureddin M, Alkhouri N. Challenges and opportunities in NASH drug development. *Nat Med* 2023.
69. Wong VW, Adams LA, de Ledinghen V, Wong GL, Sookoian S. Noninvasive biomarkers in NAFLD and NASH - current progress and future promise. *Nat Rev Gastroenterol Hepatol* 2018; **15**(8): 461-78.
70. Rinella ME, Neuschwander-Tetri BA, Siddiqui MS, et al. AASLD Practice Guidance on the clinical assessment and management of nonalcoholic fatty liver disease. *Hepatology* 2023.
71. Kugelmas M, Noureddin M, Gunn N, et al. The Use of Current Knowledge and Non-Invasive Testing Modalities for Predicting At-Risk Nonalcoholic Steatohepatitis and Assessing Fibrosis. *Liver Int* 2023.
72. Rinella ME, Dufour JF, Anstee QM, et al. Non-invasive evaluation of response to obeticholic acid in patients with NASH: Results from the REGENERATE study. *J Hepatol* 2022; **76**(3): 536-48.
73. Sanyal AJ, Miller V. Regulatory Science and Drug Approval for Alcoholic and Nonalcoholic Steatohepatitis. *Gastroenterology* 2016; **150**(8): 1723-7.
74. Masoodi M, Gastaldelli A, Hyotylainen T, et al. Metabolomics and lipidomics in NAFLD: biomarkers and non-invasive diagnostic tests. *Nat Rev Gastroenterol Hepatol* 2021; **18**(12): 835-56.
75. Loomba R. Role of imaging-based biomarkers in NAFLD: Recent advances in clinical application and future research directions. *J Hepatol* 2018; **68**(2): 296-304.
76. Stine JG, Munaganuru N, Barnard A, et al. Change in MRI-PDFF and Histologic Response in Patients With Nonalcoholic Steatohepatitis: A Systematic Review and Meta-Analysis. *Clin Gastroenterol Hepatol* 2021; **19**(11): 2274-83 e5.
77. Imajo K, Kessoku T, Honda Y, et al. Magnetic Resonance Imaging More Accurately Classifies Steatosis and Fibrosis in Patients With Nonalcoholic Fatty Liver Disease Than Transient Elastography. *Gastroenterology* 2016; **150**(3): 626-37 e7.
78. Park CC, Nguyen P, Hernandez C, et al. Magnetic Resonance Elastography vs Transient Elastography in Detection of Fibrosis and Noninvasive Measurement of Steatosis in

Patients With Biopsy-Proven Nonalcoholic Fatty Liver Disease. *Gastroenterology* 2017; **152**(3): 598-607 e2.
79. Hsu C, Caussy C, Imajo K, et al. Magnetic Resonance vs Transient Elastography Analysis of Patients With Nonalcoholic Fatty Liver Disease: A Systematic Review and Pooled Analysis of Individual Participants. *Clin Gastroenterol Hepatol* 2019; **17**(4): 630-7 e8.
80. Liang Y, Li D. Magnetic resonance elastography in staging liver fibrosis in non-alcoholic fatty liver disease: a pooled analysis of the diagnostic accuracy. *BMC Gastroenterol* 2020; **20**(1): 89.
81. Gidener T, Ahmed OT, Larson JJ, et al. Liver Stiffness by Magnetic Resonance Elastography Predicts Future Cirrhosis, Decompensation, and Death in NAFLD. *Clin Gastroenterol Hepatol* 2021; **19**(9): 1915-24 e6.
82. Ajmera V, Kim BK, Yang K, et al. Liver Stiffness on Magnetic Resonance Elastography and the MEFIB Index and Liver-Related Outcomes in Nonalcoholic Fatty Liver Disease: A Systematic Review and Meta-Analysis of Individual Participants. *Gastroenterology* 2022; **163**(4): 1079-89 e5.
83. Jayakumar S, Middleton MS, Lawitz EJ, et al. Longitudinal correlations between MRE, MRI-PDFF, and liver histology in patients with non-alcoholic steatohepatitis: Analysis of data from a phase II trial of selonsertib. *J Hepatol* 2019; **70**(1): 133-41.
84. Ravaioli F, Dajti E, Mantovani A, Newsome PN, Targher G, Colecchia A. Diagnostic accuracy of FibroScan-AST (FAST) score for the non-invasive identification of patients with fibrotic non-alcoholic steatohepatitis: a systematic review and meta-analysis. *Gut* 2023.
85. Jung J, Loomba RR, Imajo K, et al. MRE combined with FIB-4 (MEFIB) index in detection of candidates for pharmacological treatment of NASH-related fibrosis. *Gut* 2021; **70**(10): 1946-53.
86. Noureddin M, Truong E, Gornbein JA, et al. MRI-based (MAST) score accurately identifies patients with NASH and significant fibrosis. *J Hepatol* 2022; **76**(4): 781-7.
87. Kim BK, Tamaki N, Imajo K, et al. Head-to-head comparison between MEFIB, MAST, and FAST for detecting stage 2 fibrosis or higher among patients with NAFLD. *J Hepatol* 2022; **77**(6): 1482-90.
88. Ajmera V, Nguyen K, Tamaki N, Sharpton S, Bettencourt R, Loomba R. Prognostic utility of magnetic resonance elastography and MEFIB index in predicting liver-related outcomes and mortality in individuals at risk of and with nonalcoholic fatty liver disease. *Therap Adv Gastroenterol* 2022; **15**: 17562848221093869.
89. Truong E, Gornbein JA, Yang JD, et al. MRI-AST Score Accurately Predicts Major Adverse Liver Outcome, Hepatocellular Carcinoma, Liver Transplant, and Liver-Related Death. *Clin Gastroenterol Hepatol* 2023.
90. Banerjee R, Pavlides M, Tunnicliffe EM, et al. Multiparametric magnetic resonance for the non-invasive diagnosis of liver disease. *J Hepatol* 2014; **60**(1): 69-77.
91. Schaapman JJ, Tushuizen ME, Coenraad MJ, Lamb HJ. Multiparametric MRI in Patients With Nonalcoholic Fatty Liver Disease. *J Magn Reson Imaging* 2021; **53**(6): 1623-31.
92. Lee YS, Yoo YJ, Jung YK, et al. Multiparametric MR Is a Valuable Modality for Evaluating Disease Severity of Nonalcoholic Fatty Liver Disease. *Clin Transl Gastroenterol* 2020; **11**(4): e00157.
93. Troelstra MA, Witjes JJ, van Dijk AM, et al. Assessment of Imaging Modalities Against Liver Biopsy in Nonalcoholic Fatty Liver Disease: The Amsterdam NAFLD-NASH Cohort. *J Magn Reson Imaging* 2021; **54**(6): 1937-49.
94. Andersson A, Kelly M, Imajo K, et al. Clinical Utility of Magnetic Resonance Imaging Biomarkers for Identifying Nonalcoholic Steatohepatitis Patients at High Risk of Progression: A Multicenter Pooled Data and Meta-Analysis. *Clin Gastroenterol Hepatol* 2022; **20**(11): 2451-61 e3.

95. Dennis A, Kelly MD, Fernandes C, et al. Correlations Between MRI Biomarkers PDFF and cT1 With Histopathological Features of Non-Alcoholic Steatohepatitis. *Front Endocrinol (Lausanne)* 2020; **11**: 575843.
96. Blake L, Duarte RV, Cummins C. Decision analytic model of the diagnostic pathways for patients with suspected non-alcoholic fatty liver disease using non-invasive transient elastography and multiparametric magnetic resonance imaging. *BMJ open* 2016; **6**(9): e010507.
97. Eddowes PJ, McDonald N, Davies N, et al. Utility and cost evaluation of multiparametric magnetic resonance imaging for the assessment of non-alcoholic fatty liver disease. *Aliment Pharmacol Ther* 2018; **47**(5): 631-44.
98. Ahn JC, Connell A, Simonetto DA, Hughes C, Shah VH. Application of Artificial Intelligence for the Diagnosis and Treatment of Liver Diseases. *Hepatology* 2021; **73**(6): 2546-63.
99. Listopad S, Magnan C, Asghar A, et al. Differentiating between liver diseases by applying multiclass machine learning approaches to transcriptomics of liver tissue or blood-based samples. *JHEP Rep* 2022; **4**(10): 100560.
100. Francque S, Szabo G, Abdelmalek MF, et al. Nonalcoholic steatohepatitis: the role of peroxisome proliferator-activated receptors. *Nat Rev Gastroenterol Hepatol* 2021; **18**(1): 24-39.
101. Tyagi S, Gupta P, Saini AS, Kaushal C, Sharma S. The peroxisome proliferator-activated receptor: A family of nuclear receptors role in various diseases. *Journal of advanced pharmaceutical technology & research* 2011; **2**(4): 236-40.
102. Oliveira AC, Bertollo CM, Rocha LT, Nascimento EB, Jr., Costa KA, Coelho MM. Antinociceptive and antiedematogenic activities of fenofibrate, an agonist of PPAR alpha, and pioglitazone, an agonist of PPAR gamma. *European journal of pharmacology* 2007; **561**(1-3): 194-201.
103. Gale EA. Lessons from the glitazones: a story of drug development. *Lancet* 2001; **357**(9271): 1870-5.
104. DeFronzo RA, Inzucchi S, Abdul-Ghani M, Nissen SE. Pioglitazone: The forgotten, cost-effective cardioprotective drug for type 2 diabetes. *Diabetes & vascular disease research* 2019; **16**(2): 133-43.
105. Cusi K, Orsak B, Bril F, et al. Long-Term Pioglitazone Treatment for Patients With Nonalcoholic Steatohepatitis and Prediabetes or Type 2 Diabetes Mellitus: A Randomized Trial. *Ann Intern Med* 2016; **165**(5): 305-15.
106. Mantovani A, Byrne CD, Scorletti E, Mantzoros CS, Targher G. Efficacy and safety of anti-hyperglycaemic drugs in patients with non-alcoholic fatty liver disease with or without diabetes: An updated systematic review of randomized controlled trials. *Diabetes Metab* 2020; **46**(6): 427-41.
107. Mantovani A, Byrne CD, Targher G. Efficacy of peroxisome proliferator-activated receptor agonists, glucagon-like peptide-1 receptor agonists, or sodium-glucose cotransporter-2 inhibitors for treatment of non-alcoholic fatty liver disease: a systematic review. *Lancet Gastroenterol Hepatol* 2022; **7**(4): 367-78.
108. Guixé-Muntet S, Biquard L, Szabo G, et al. Review article: vascular effects of PPARs in the context of NASH. *Aliment Pharmacol Ther* 2022; **56**(2): 209-23.
109. Maeda N, Takahashi M, Funahashi T, et al. PPARgamma ligands increase expression and plasma concentrations of adiponectin, an adipose-derived protein. *Diabetes* 2001; **50**(9): 2094-9.
110. Francque SM, Bedossa P, Ratziu V, et al. A Randomized, Controlled Trial of the Pan-PPAR Agonist Lanifibranor in NASH. *N Engl J Med* 2021; **385**(17): 1547-58.

111. Gawrieh S, Noureddin M, Loo N, et al. Saroglitazar, a PPAR-alpha/gamma Agonist, for Treatment of NAFLD: A Randomized Controlled Double-Blind Phase 2 Trial. *Hepatology* 2021; **74**(4): 1809-24.
112. Cariou B, Charbonnel B, Staels B. Thiazolidinediones and PPARγ agonists: time for a reassessment. *Trends in Endocrinology & Metabolism* 2012; **23**(5): 205-15.
113. Alberti KG, Eckel RH, Grundy SM, et al. Harmonizing the metabolic syndrome: a joint interim statement of the International Diabetes Federation Task Force on Epidemiology and Prevention; National Heart, Lung, and Blood Institute; American Heart Association; World Heart Federation; International Atherosclerosis Society; and international association for the Study of Obesity. *Circulation* 2009; **120**(16): 1640-5.
114. Austin MA, King MC, Vranizan KM, Krauss RM. Atherogenic lipoprotein phenotype. A proposed genetic marker for coronary heart disease risk. *Circulation* 1990; **82**(2): 495-506.
115. Malik A, Nadeem M, Malik MI. Efficacy of elafibranor in patients with liver abnormalities especially non-alcoholic steatohepatitis: a systematic review and meta-analysis. *Clinical journal of gastroenterology* 2021; **14**(6): 1579-86.
116. Targher G, Mantovani A, Byrne CD. Mechanisms and possible hepatoprotective effects of glucagon-like peptide-1 receptor agonists and other incretin receptor agonists in non-alcoholic fatty liver disease. *Lancet Gastroenterol Hepatol* 2023; **8**(2): 179-91.
117. Loomba R, Abdelmalek MF, Armstrong MJ, et al. Semaglutide 2.4 mg once weekly in patients with non-alcoholic steatohepatitis-related cirrhosis: a randomised, placebo-controlled phase 2 trial. *Lancet Gastroenterol Hepatol* 2023; **8**(6): 511-22.
118. Abdelmalek M, Choi J, Kim Y, Seo K, Hompesch M, Baek S. HM15211, a novel GLP-1/GIP/Glucagon triple-receptor co-agonist significantly reduces liver fat and body weight in obese subjects with non-alcoholic fatty liver disease: A Phase 1b/2a, multi-center, randomized, placebo-controlled trial. *Journal of Hepatology* 2020; **73**: S124.
119. Müller TD, Finan B, Clemmensen C, DiMarchi RD, Tschöp MH. The New Biology and Pharmacology of Glucagon. *Physiological reviews* 2017; **97**(2): 721-66.
120. Hager J, Hansen L, Vaisse C, et al. A missense mutation in the glucagon receptor gene is associated with non-insulin-dependent diabetes mellitus. *Nature genetics* 1995; **9**(3): 299-304.
121. Unger RH. Glucagon and the Insulin:Glucagon Ratio in Diabetes and Other Catabolic Illnesses. *Diabetes* 1971; **20**(12): 834-8.
122. Williams JF, Jr., Childress RH, Chip JN, Border JF. Hemodynamic effects of glucagon in patients with heart disease. *Circulation* 1969; **39**(1): 38-47.
123. Altimmune Announces Significant Reductions In Liver Fat Content And Body Weight In 12-Week Phase 1b Clinical Trial Of Pemvidutide In Subjects With NAFLD. *https://iraltimmunecom/news-releases/news-release-details/altimmune-announces-significant-reductions-liver-fat-content-and* 2022.
124. Plutzky J, Harris MSS, Alonso C, et al. Abstract 13440: Effect of Pemvidutide (ALT-801), a Novel GLP-1/Glucagon Dual Receptor Agonist, on Pathogenic Lipid Mediators. *Circulation* 2022; **146**(Suppl_1): A13440-A.