Table 1. Clinical and serological characteristics of the control and patient population.

	Controls	Not NASH	NASH	P value*
Number (n)	21	48	65	
Age (years)	44.8±9.0	50.4±13.6	51.4±12.8	0.381
Gender (male; female)	14;7	26;22	36;29	0.328
Body mass index (kg/m^2)	21.9±2.6	27.9±5.3	29.1±5.1	0.046
Visceral fat area (cm^2)		141.1±34.9	151.4±40.4	0.171
Subcutaneous fat area (cm^2)		191.3±54.1	201.9±58.1	0.173
Fasting blood sugar (mg/dl)	84.2±10.1	101.2±25.1	106.2±29.4	0.301
AST (IU/l)	23.8±4.8	40.1±16.1	41.5±17.1	0.284
ALT (IU/l)	22.8±6.2	49.3±27.2	54.3±26.9	0.212
C-reactive protein (mg/l)	0.27±0.21	0.73±0.47	1.35±0.94	0.011
HOMA-IR	0.96±0.17	2.61±1.39	3.66±2.01	0.009
Dyslipidemia (%)	0	17 (35.1)	23 (47.9)	0.052
Hypertension (%)	0	19 (39.5)	20 (41.5)	0.522
Steatosis grade				0.003
5–33%		22	23	
33–66%		19	31	
>66%		7	11	
Lobular inflammation				7×10^{-12}
None		15	0	
<2 foci per 200x field		23	31	
2–4 foci per 200x field		8	22	
>4 foci per 200x field		2	12	
Liver cell ballooning				3×10^{-16}
None		30	0	
Few balloon cells		16	47	
Many balloon cells		2	18	
Fibrosis stage				3×10^{-16}
None		21	0	
Perisinusoidal or periportal		23	38	
Perisinusoidal and portal/periportal		4	20	
Bridging fibrosis		0	5	
Cirrhosis		0	2	

Numbers represent the mean ± SD. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model for the assessment of insulin resistance. P values correspond to the comparison of the three subjects groups (not NASH, borderline NASH and definite NASH) using the Kruskal–Wallis tests for continuous factors.

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mia, hypertension, BMI, VFA, and SFA. To our knowledge, this is the first report to show that sCD14 in blood is a promising biomarker for diagnosis of NASH and assessing liver inflammation in NASH.

NAFLD, which can progress to steatohepatitis and cirrhosis, is perhaps the most common type of chronic liver disease in obese patients [4–5]. As effective therapies for NAFLD have not yet been established, the identification of risk factors for disease progression, such as severe liver inflammation, would help to guide the implementation of risk-reduction strategies for these patients [25]. However, discrimination between mild and severe liver inflammation in patients with NASH is very difficult if using imaging modalities alone [26]. Indeed, liver biopsy examination is currently the only method that can precisely diagnose NASH and evaluate liver inflammation in patients with NASH [27]. However, liver biopsy is invasive, expensive, and is associated with a relatively high risk of complications [14]. Moreover, the accuracy of the procedure used to assess the severity of liver fibrosis is questionable because of intra- and inter-observer variation [27–31]. Sampling error has also been reported, even in patients with NASH [32]. Therefore, a non-invasive, reproducible, conceptually simple, and highly reliable test is needed to diagnose NASH and evaluate the severity of liver inflammation in patients with NASH.

CD14 is an effective mediator for the activation of monocytes in response to bacterial endotoxins. The serum sCD14 levels increase during the systemic response to bacterial invasion and endotoxin. Actually, we showed that sCD14 was increased in the culture medium of RAW264.7 cells after LPS treatment, suggesting that sCD14 may be shed from mCD14 in RAW264.7 cells under the effect of LPS. Here, we presented a hypothesis that the increased sCD14 levels in patients with NASH might reflect the severity of

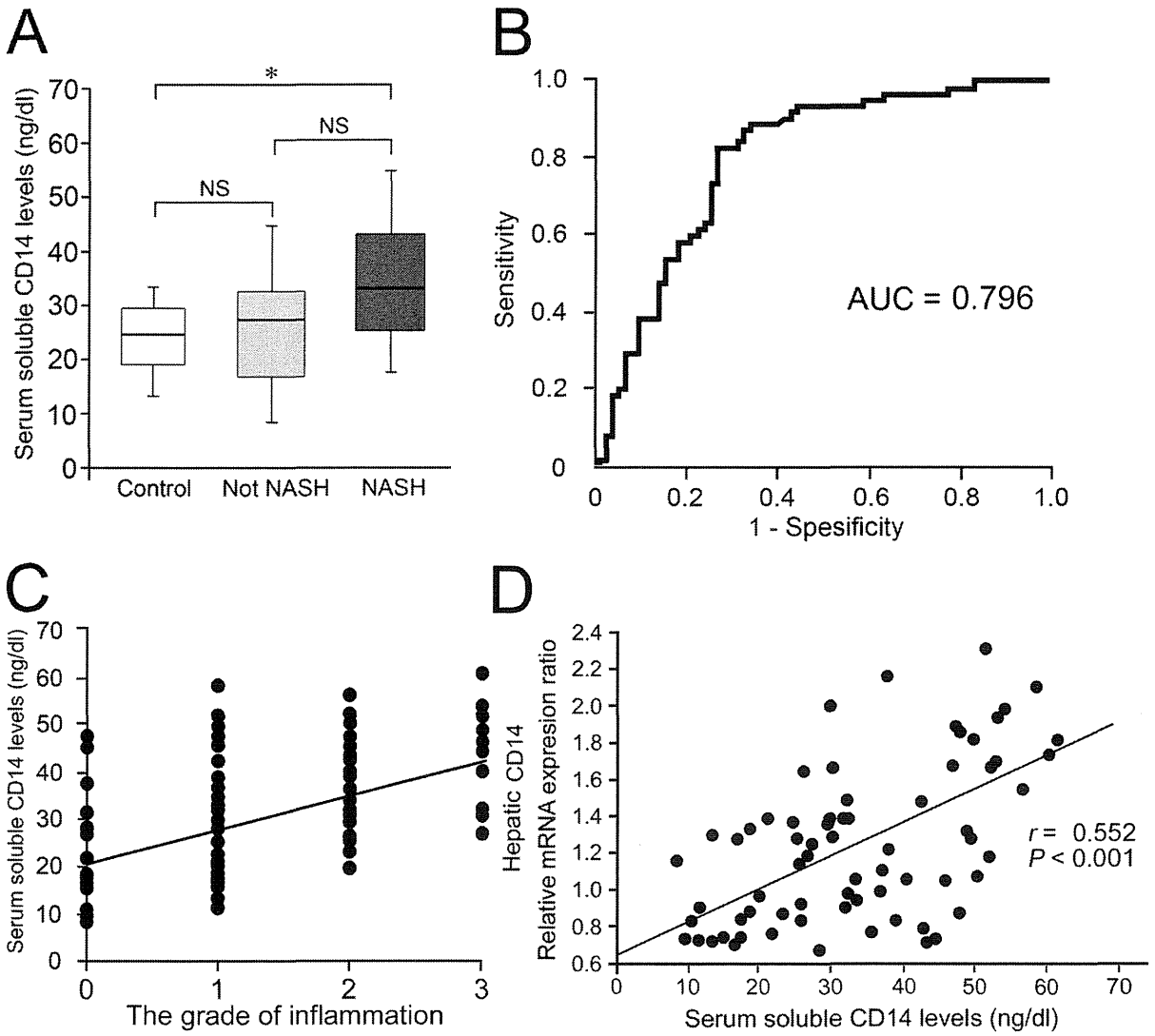

Figure 1. Serum sCD14 levels, liver inflammation and hepatic CD14 expression. (A) Serum sCD14 levels in control subjects and patients with NAFLD. The graph shows the interquartile range (box), median (the line), and range (lines) of serum sCD14 levels. The median (range) values (ng/dl) are 24.3 (13.3–32.4), 27.5 (9.21–44.2), and 32.2 (17.9–54.2) for control subjects ($n = 21$), not NASH ($n = 48$), and NASH ($n = 65$), respectively. Statistical significance was determined by analysis of variance with Scheffe's correction for multiple testing. *$P<0.05$. (B) Receiver operating characteristic (ROC) curve and area under the ROC curve (AUROC) for distinguishing between not NASH (n = 48) including control subjects (n = 21) and NASH (n = 65) using serum sCD14 level. (C) Relationship between serum sCD14 and the grade of liver inflammation in patients with NAFLD. Serum sCD14 levels are significantly correlated with the grade of liver inflammation (Spearman's $r = 0.498$, $P<0.001$). (D) Relationship between serum sCD14 and hepatic CD14 mRNA expression in patients with NAFLD. Serum sCD14 levels are significantly correlated with hepatic CD14 mRNA expression levels (Spearman's $r = 0.552$, $P<0.001$). The correlation was determined in 69 patients with NAFLD patients.
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liver inflammation. Consistent with this hypothesis, we observed as significant association between serum sCD14 levels and definite NASH or the grade of liver inflammation in histological sections in liver biopsy-confirmed NAFLD. Furthermore, our previous report showed that leptin-induced upregulation of hepatic CD14 and the resulting hyper-reactivity to low-dose LPS during NASH progression were closely associated with increased liver inflammation [15]. These results were confirmed by the observation that hepatic CD14 expression was much higher in patients with NASH than in healthy controls and patients with nonalcoholic fatty liver [15]. In the present study, serum sCD14 levels were positively correlated with hepatic CD14 expression levels in patients with NAFLD. These results suggest that serum sCD14 levels might increase following increasing liver inflammation in NAFLD, reflecting increased hepatic CD14 expression. In other words, the sCD14 is likely to be liver CD14 that is shed into the blood.

Determination of the severity of liver inflammation is important to evaluate the prognosis of patients with NASH. To explore the clinical usefulness of sCD14 as a biomarker for liver inflammation, we investigated the diagnostic ability of serum sCD14 levels using multiple regression analysis and ROC curves. We found that serum sCD14 levels are independently associated with increased risk of severe liver inflammation in NAFLD patients. In addition, we found that a serum sCD14 cutoff level of 29.5 ng/dl showed good sensitivity and specificity for liver inflammation in patients with NAFLD, with values of 78.2 and 72.4%. The resulting AUROC was 0.752, indicating moderate accuracy. These results indicate that the serum sCD14 level is a good biomarker for liver

Table 2. Correlations between serum sCD14 level and clinical parameters.

Factor	rho	P-value
Age (years)	−0.082	0.871
Body mass index (kg/m^2)	0.021	0.522
Visceral fat area (cm^2)	0.123	0.354
Subcutaneous fat area (cm^2)	−0.053	0.517
Fasting Blood Sugar (mg/dl)	0.105	0.188
AST (IU/l)	0.136	0.153
ALT (IU/l)	0.214	0.049
C-reactive protein (mg/l)	0.223	0.047
HOMA-IR	0.217	0.052
NAS	0.354	0.004
Steatosis	−0.042	0.492
Inflammation	0.498	<0.001
Ballooning	0.274	0.051
Fibrosis	0.365	<0.001

Numbers represent the mean ± SD. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model for the assessment of insulin resistance; NAS, NAFLD activity score. The correlation between serum sCD14 levels and other parameters is examined by Spearman correlations coefficient.
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inflammation in patients with NAFLD. A previous report showed that serum sCD14 levels in patients with NASH increased with increasing fibrosis stage; however, that report did not evaluate liver inflammation [33]. By contrast, we showed that serum sCD14 levels are strongly correlated with the grade of liver inflammation but not the stage of liver fibrosis. The serum sCD14 levels were also positively correlated with hepatic CD14 expression levels in patients with NAFLD. These results suggest that increased serum sCD14 levels reflect liver inflammation in NAFLD patients. Similarly, previous report showed that circulating microparticles from CD14 positive cells were correlated with severity of liver inflammation in patients with NAFLD [34]. However, we believe that sCD14 is a very convenient tool for evaluation of liver inflammation grade when compared with microparticles.

Table 4. Multiple logistic regression analysis of factors associated with grade 2–3 liver inflammation compared to grade 0–1 liver inflammation in NAFLD patients.

Factor	Odds ratio	95% CI	P value
Age (years)	1.071	0.992–1.149	0.0729
Gender	1.976	0.241–17.49	0.5287
Body mass index (kg/m^2)	1.110	0.881–1.329	0.3987
ALT (IU/l)	0.995	0.938–1.029	0.2756
C-reactive protein (mg/l)	1.395	0.827–2.339	0.2131
sCD14 (ng/dl)	8.853	1.221–63.08	0.0116*

Abbreviations: ALT, alanine aminotransferase; sCD14, soluble CD14.
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Several limitations of our study should be discussed. First, we did not conduct liver biopsies in the healthy control group for ethical reasons. Second, some patient selection bias may exist because liver biopsy may have been reserved for patients with NAFLD who were deemed likely to have NASH. Third, using liver biopsy as the 'gold standard' for assessing the accuracy of sCD14 has important limitations associated with sampling errors, as well as intra- and inter-observer variability, which are at least partly linked to the biopsy size [32]. Finally, serum sCD14 levels may increase in other conditions such as cholestasis, biliary atresia, and ischemia reperfusion injury [35–36]. However, these are extremely unusual conditions.

In conclusion, we confirmed that serum sCD14 may be a useful and non-invasive biomarker for diagnosis of NASH and assessing liver inflammation in patients with NAFLD, who are at high risk of progressing to advanced liver fibrosis. Further research, including larger-scale clinical studies or combination of serum sCD14 and other non-invasive biomarkers of NASH such as CK18, are

Table 3. Clinical and serological characteristics of NAFLD patients with mild and severe liver inflammation.

	Grade 0–1 liver inflammation	Grade 2–3 liver inflammation	P value*
Number (n)	43	70	
Age (years)	47.2±13.2	52.3±12.9	0.046
Gender (male; female)	23;20	43;27	0.037
Body mass index (kg/m^2)	27.9±5.3	29.9±5.9	0.042
Visceral fat area (cm^2)	140.7±35.1	149.8±46.2	0.051
Subcutaneous fat area (cm^2)	199.5±44.9	191.9±48.1	0.226
Fasting Blood Sugar (mg/dl)	105.2±13.1	110.2±13.4	0.251
AST (IU/l)	42.3±14.1	43.2±14.3	0.430
ALT (IU/l)	45.5±12.9	57.1±17.6	0.048
C-reactive protein (mg/l)	0.73±0.46	1.18±0.98	0.043
HOMA-IR	3.43±1.33	3.59±1.31	0.431
sCD14 (ng/dl)	25.7±10.5	31.2±11.6	0.009

Numbers represent the mean ± SD. Abbreviations: AST, aspartate aminotransferase; ALT, alanine aminotransferase; HOMA-IR, homeostasis model for the assessment of insulin resistance. P values correspond to the comparison between grade 0–1 liver inflammation and grade 2–3 liver inflammation in NAFLD patients using the Student's t-test for continuous factors.
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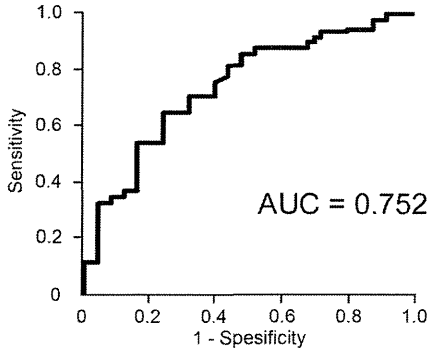

Inflammaroty grade	Cut off value	Se (%)	Sp (%)	PPV (%)	NPV (%)
Grade ≧ 2	29.5 (ng/dl)	78.2	72.4	79.6	62.9

Figure 2. Serum sCD14 levels for diagnosis of the grade of liver inflammation. Receiver operating characteristic (ROC) curve and area under the ROC curve (AUROC) for discriminating between patients with severe (grade 2–3) or mild (grade 0–1) liver inflammation using serum sCD14 levels in 113 patients are shown. Serum sCD14 levels can diagnose severe liver inflammation in patients with NAFLD with moderate accuracy.
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needed to fully investigate the diagnostic and therapeutic implications of our findings.

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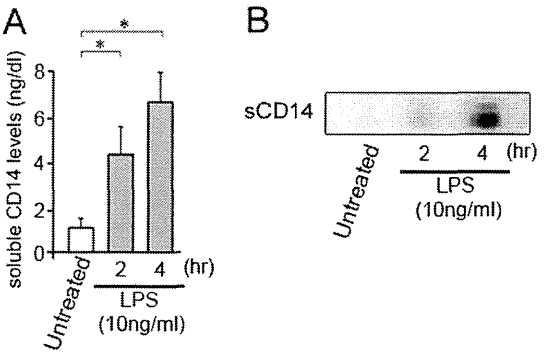

Figure 3. Lipopolysaccharide (LPS) increases sCD14 *in vitro*. sCD14 in cell culture medium from sham- and LPS-treated RAW264.7 cells was compared by (A) Western immunoblot analysis and (B) a sandwich enzyme-linked immunosorbent assay. LPS increased sCD14 in cell culture medium from RAW 264.7 cells. The immunoblot is representative of three independent experiments. Results are presented as means ± SD. Statistical significance was determined using ANOVA with Scheffe's multiple testing correction (*p value <0.05).
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Author Contributions

Conceived and designed the experiments: AN YO KI MY. Performed the experiments: YO KI. Analyzed the data: YO KI TK WT YS SK HM SM YN KF HK. Contributed reagents/materials/analysis tools: KW SS. Wrote the paper: AN YO KI MY.

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EXPERT OPINION

1. Background
2. Medical need
3. Existing treatment
4. Market review
5. Current research goals
6. Scientific rationale
7. Competitive environment
8. Potential development issues
9. Conclusion
10. Expert opinion

Emerging drugs for non-alcoholic steatohepatitis

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Introduction: The prevalence of non-alcoholic fatty liver disease (NAFLD) and non-alcoholic steatohepatitis (NASH) is increasing along with the worldwide epidemic of obesity and their strong association with metabolic syndrome. Currently existing pharmacological therapies include anti-oxidants, insulin-sensitizing agents, lipid-lowering drugs and cytoprotective agents, but there is a lack of consensus regarding the most effective and appropriate pharmacologic therapies for NASH. Clinical trials examining new therapeutic drugs for NASH that act via various mechanisms are being performed in several countries, and these drugs may strongly influence current NASH treatment.
Areas covered: This article provides a review of recent data on the safety and efficacy of existing and emerging agents for the treatment of NASH.
Expert opinion: Ideally, treatment for NASH should not only improve liver disease, but also reduce the risks of adverse cardiovascular outcomes and the development of diabetes and cancers. However, this goal is likely to be too high in the context of clinical trials designed to obtain approval for the treatment of liver disease. The only way to achieve the goal is to accumulate the results of these relatively short-term clinical trials.

Keywords: clinical trial, non-alcoholic fatty liver disease, non-alcoholic steatohepatitis, pharmacologic therapy

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1. Background

Non-alcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury in many countries around the world [1]. NAFLD represents a spectrum of conditions that are characterized histologically by macrovesicular hepatic steatosis, and a diagnosis is made after excluding a history of the consumption of alcohol in amounts sufficient to be considered harmful to the liver. The histologic changes range over a wide spectrum, extending from simple steatosis to non-alcoholic steatohepatitis (NASH) and liver cirrhosis [1,2]. The prevalence of NASH in the general population ranges from 1 to 5%, while that of NAFLD ranges from 15 to 39% [3]. The prevalence of both of these conditions is escalating given the worldwide epidemic of obesity and their strong association with metabolic syndrome.

NASH, unlike simple steatosis, is a potentially progressive disease. Without appropriate treatment, NASH can progress to cirrhosis, decompensated liver disease and hepatocellular carcinoma [2,4]. Furthermore, NASH is predicted to become the leading cause of liver transplantation in the USA by 2020 [5]. This review focuses on the clinical development of several emerging drugs for the treatment of NASH and the likely impact of these drugs on current treatment standards.

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2. Medical need

Despite strong efforts and numerous clinical trials, a definitive treatment for NASH has not yet been established. Lifestyle changes (weight loss and exercise) are the best preventive and curative measures for NASH and metabolic syndrome, which is associated with NAFLD/NASH [6]; however, such changes cannot be effectively instituted in the majority of patients, who require supportive pharmacological therapies.

Because of the large population at risk, a dramatic increase in healthcare spending for the treatment of NAFLD/NASH and metabolic syndrome can be expected [7]. Therefore, the development of effective pharmacological therapies is also urgently needed from a financial aspect.

3. Existing treatment

The ideal treatment for NAFLD/NASH patients has not yet been established, and most current therapeutic trials lack sufficient power to show a definitive benefit. Thus, current treatments are primarily directed toward cardiovascular risk reduction and improving the metabolic variables that contribute to disease progression. More focused therapy and scheduled follow-up examinations are warranted in patients with NASH because they are at increased risk of progression to cirrhosis, decompensated liver disease and hepatocellular carcinoma. Approaches to the management of NASH can be divided into lifestyle modifications, pharmacological therapies and surgical interventions. Current existing pharmacological therapies include anti-oxidants, insulin-sensitizing agents, lipid-lowering drugs and cytoprotective agents. The authors have summarized the current existing pharmacological therapies (described in this section) and the emerging drugs (described in the Sections 7 and 8) in Table 1.

3.1 Lifestyle modifications

Weight reduction by diet and exercise is generally recommended as an initial step in the management of NAFLD/NASH. Effective and sustained weight loss is associated with a marked improvement in liver enzymes and stable or improved liver histology [8]. A randomized controlled trial showed the efficacy of lifestyle interventions using a combination of diet, exercise and behavior modification, with a weight reduction goal of 7 – 10%, on the clinical parameters of NASH [6]. In this trial, the participants assigned to the lifestyle intervention for 48 weeks lost an average of 9.3% of their weight versus only 0.2% in the control group, and weight loss goals were associated with significant improvements in steatosis and lobular inflammation, but not with any changes in fibrosis. Furthermore, an earlier study demonstrated that a body weight loss of even 5% is associated with the improvement and normalization of liver enzymes in patients with NAFLD [9]. The most difficult problem associated with this intervention was the fact that only about 40% of patients who were enrolled were able to achieve the necessary weight loss goal, despite aggressive dietary counseling and exercise recommendations [10].

3.2 Anti-oxidants

Oxidative stress is thought to contribute to the pathogenesis of NAFLD/NASH, and several studies have tested the efficacies of anti-oxidants. The rationale behind this potential therapy is to reduce the generation of reactive oxygen species (ROS) in the liver and to inhibit the activation of inflammatory cytokines involved in steatohepatitis [11].

The effect of vitamin E (α-tocopherol) has been extensively examined in children and adults. The largest trial of vitamin E to date examined the effect of 96 weeks of vitamin E therapy (at a dose of 800 mg/day) in non-diabetic patients with biopsy-proven NASH and showed a significant improvement in steatosis and lobular inflammation, compared with a placebo [12]. No improvement in fibrosis was seen in this trial, although smaller trials have shown modest improvements in fibrosis in response to vitamin E and C combination therapy [13]. Based on the results of the former trial in non-diabetic patients with biopsy-proven NASH, the guidelines of the American Association for the Study of Liver Disease (AASLD) [14] recommend that vitamin E prescription only be used in non-diabetic patients with biopsy-proven NASH, and not in NASH patients with diabetes or NAFLD patients who have not undergone a liver biopsy. Liver biopsy is an invasive and somewhat painful procedure and may be associated with life-threatening complications in some individuals [15]. Therefore, many NAFLD patients do not undergo a liver biopsy. Furthermore, diabetes is one of the most important factors associated with advanced fibrosis [16] and the progression of fibrosis [17] and is a strong predictor of poor survival among patients with NAFLD [18] as well as increasing the risk for hepatocellular carcinoma [19]. Accordingly, the AASLD guidelines actually do not recommend vitamin E for patients who might be expected to derive the most benefit from this treatment.

Although vitamin E is generally thought of as a treatment without side effect, a study unrelated to NASH/NAFLD research has shown that high doses of vitamin E greater than 400 mg daily may be associated with increased mortality [20]. Ultimately, vitamin E at a dose of 800 mg once daily seems reasonable to offer adult NASH patients, but the other potential health risks should be discussed with the patient.

3.3 Thiazolidinediones

Thiazolidinediones (TZDs) increase insulin sensitivity through their action as peroxisome proliferator-activated receptor γ (PPARγ) agonists. They improve peripheral and hepatic insulin sensitivity by promoting the redistribution of fat from the liver and muscle to adipose tissue and by increasing the circulating levels of adiponectin [21]. Pioglitazone and rosiglitazone are the two TZDs that are investigated most often; however, because of an increased risk of cardiovascular disease

Table 1. Currently existing pharmacological therapies and emerging drugs for NASH.

	Existing treatments	Emerging drugs	
		In clinical trials	In preclinical stage
Insulin-sensitizing agents	Metformin, pioglitazone	OCA GFT-505 Oltipraz EPA	
Anti-oxidants	Vitamin E	DR cysteamine Mitoquinone EPA	
Cytoprotective agents	UDCA	OCA	
Antidiabetic agents (excluding insulin-sensitizing agents)		BLX-1002	Ipragliflozin Sitagliptin
Lipid-lowering agents	Statins, ezetimibe	OCA GFT-505 EPA	ISIS-DGAT2Rx PYN22 Nanoveson
Others		Anti-CD3 monoclonal antibody	Anti-VEGFR2 antibody GR-MD-02

Drugs with multiple pharmacological actions are listed in each relevant section.
DR cysteamine: Delayed-release cysteamine; EPA: Eicosapentaenoic acid; NASH: Non-alcoholic steatohepatitis; OCA: Obeticholic acid; UDCA: Ursodeoxycholic acid.

(CVD), the use of rosiglitazone is restricted. Rosiglitazone has been withdrawn from the European market according to a recommendation made by the European Medicines Agency (EMA), and its use has been greatly restricted in the USA based on the recommendation of the US Food and Drug Administration (FDA).

Pioglitazone has caused a significant improvement in both histologic and biochemistry markers of NASH in some randomized controlled trials, regardless of glucose tolerance [12,22,23]. The dose of pioglitazone was 30 [12,23] or 45 mg/day [22] in these studies.

Despite the majority of studies showing that the normalization of aminotransferase levels and improvements in steatosis and inflammation are achievable with pioglitazone, an improvement in fibrosis is unlikely [12,22]. In addition, adequate diet, exercise and behavior modification interventions must be performed in conjunction with this pharmacological therapy, and if excessive weight gain is seen, the curative effect is questionable. Furthermore, pioglitazone should be avoided in patients with heart failure or a history of bladder cancer. This drug has been withdrawn from the market in France and its use is very restricted in Germany and the USA because the use of pioglitazone for > 2 years has been reported to be weakly associated with an increased risk of bladder cancer [24].

3.4 Metformin

Metformin belongs to the biguanide class of medications that has been used to control type 2 diabetes mellitus. Metformin increases insulin sensitivity by decreasing hepatic gluconeogenesis and limiting triglyceride production [25]. This potential mechanism is the basis for using this drug as a pharmacotherapy for NAFLD/NASH. Metformin has shown great promise in improving steatohepatitis in animal models [26], and improved serum aminotransferases and metabolic parameters have been seen in pilot human studies [27]. Unfortunately, recent randomized controlled trials have not shown a histologic benefit of metformin therapy for NASH in either adult or pediatric patient populations [28,29]. However, because of the high prevalence of coexisting type 2 diabetes, its excellent tolerance and safety profiles and its ability to initiate weight loss (which may itself account for many of the observed beneficial effects), metformin remains a commonly prescribed agent in patients with NASH.

3.5 Statins

Statins prevent cholesterol synthesis by inhibiting the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase and are an important class of agents for the treatment of dyslipidemia. Patients with NAFLD and NASH have an increased risk for CVD, and several studies have established CVD as the most common cause of death [30]. Patients with NAFLD/NASH should be risk-stratified for CVD, and their cardiovascular risk factors should be managed accordingly. Thus, the treatment of dyslipidemia should be considered in the overall frame work of cardiovascular risk reduction in patients with NAFLD/NASH [31].

Trials to date have produced mixed results for the effects of statins on transaminases and histology in patients with NASH [32-34], and their role has not yet been established. Statins can induce increases in aminotransferase levels, necessitating drug discontinuation, but they can be used to treat underlying hepatic steatosis. These drugs decrease fat, both peripherally and viscerally, specifically in the liver. When fat is removed from the liver, short-term increases in aminotransferase levels can occur. These transient increases should not progress to liver damage, and fat removal should eventually lead to improved aminotransferase levels.

The AASLD has recommended against the use of statins for the treatment of NASH until randomized controlled trials prove their histologic efficacy [14]. Considering the link between NAFLD/NASH and CVD, the efficacy of statins in patients with NAFLD/NASH might be best measured as the reduction in major adverse cardiovascular events, rather than histologic data. The overall benefit of statins in patients with NASH may outweigh the current lack of specific histologic improvement.

3.6 Ezetimibe

Ezetimibe is a newer agent that lowers lipid levels by inhibiting cholesterol absorption. Studies using animal models have shown improvements in hepatic steatosis, necroinflammation and fibrosis when used in combination with acarbose [35]. The efficacy of ezetimibe for the treatment of NASH has been demonstrated in a pilot study [36], and another recent study showed improvements in hepatic steatosis, necroinflammation and metabolic parameters in 45 patients with NAFLD who were treated for 24 months with ezetimibe, although no significant change in fibrosis was noted [37]. Confirmation in larger placebo-controlled trials is required for patients with NASH.

3.7 Ursodeoxycholic acid

Medications classified as cytoprotective agents prevent apoptosis and downregulate the inflammatory cascade, two mechanisms that are thought to play central roles in the pathogenesis of NASH. Ursodeoxycholic acid (UDCA) is a prime example of a cytoprotective agent that has been investigated for the treatment of NAFLD/NASH. Two well-designed randomized controlled trials examining UDCA failed to show any significant histological improvements with UDCA alone in patients with biopsy-proven NASH [38,39]. These findings suggested that the biochemical and histological improvements previously observed using a combination of UDCA and vitamin E therapy [40,41] might have been largely attributable to the vitamin E.

4. Market review

The NASH market was estimated to be worth US$500 million in 2009. The major reason for the limited market revenues is that there are no approved drugs for the treatment of NASH. As mentioned above, anti-oxidants, antidiabetic medications and antihyperlipidemics are some of the off-label products that are currently used by NASH patients. The modest growth forecast can be primarily attributed to a weak pipeline landscape. Because low diagnosis rates, low prescription rates and low treatment-seeking rates continue to pose significant challenges for prospective market entrants, the market landscape in the future is expected to remain static without any significant dynamism. Overall, between 2009 and 2017, the NASH market is expected to grow at a compound annual growth rate (CAGR) of 16%, reaching US$1.65 billion by 2017 [42]. This growth will be mainly driven by the increasing incidence and prevalence of NASH, which are expected to lead to improvements in diagnosis and treatment rates.

The NASH market poses huge unmet needs. This is due to lack of disease understanding and low diagnosis rates, though it is estimated that more NASH patients exist potentially, with increase in prevalence of diabetes, obesity and hyperlipidemia. Thus, NASH continues to present opportunities for strong pipeline candidates. However, to capture the high unmet needs posed by the existing market, companies will need to overcome the prevailing product weakness and the adverse effects of the off-label products that are presently being used for the treatment of NASH.

5. Current research goals

Direct comparisons between therapeutic options remain a challenge as a result of the mechanistic diversity of therapies and the heterogeneity of methods and study end points used in NASH trials to date. The introduction of Kleiner's NAFLD Activity Score (NAS) [43] has resulted in a degree of uniformity in histological analyses. However, the extent to which an improvement in the NAS reflects a clinically significant decrease in the risk of developing cirrhosis or liver-related mortality remains uncertain. Furthermore, the potential impacts of the improvement of various components of the NAS, such as steatosis versus inflammation, on the risk of developing cirrhosis also remain unknown. With disease progression to cirrhosis, active steatohepatitis lesions may also decrease. Therefore, if an improvement in the NAS is used as a primary end point, an improvement by a minimum of two points with contributions from more than one parameter and no worsening of fibrosis, should be used to maximize the clinical relevance and robustness of the findings [44].

An evaluation of the severity of fibrosis is not included in the NAS, so the progression or regression of fibrosis can be used as a secondary end point. Transient elastography can reportedly be used to predict the severity of fibrosis in patients with NAFLD [45]. This tool is non-invasive and easy to use, making it useful for monitoring the severity of hepatic fibrosis in patients with NAFLD [46]. Magnetic resonance (MR) elastography [47] is also a non-invasive tool that is expected to be useful for the diagnosis of fibrosis severity.

For short-term studies (Phase I and IIa) designed mainly to assess the tolerability of new drugs and to look for futility signals to direct decisions regarding further development, an improvement in hepatic steatosis, as determined using MR spectroscopy, and a sustained improvement in the aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels may be useful as efficacy end points [37]. However, the observations that both NAFLD and NASH may exist without an elevation in the serum ALT value and that patients with normal ALT values may also have NASH with severe fibrosis must be considered [48].

There are many uncertainties in the diagnostic approaches, evaluation and management of NAFLD/NASH. Diagnosis

Table 2. List of some NASH drugs currently in development.

Compound	Company	Stage of development	Mechanism of action
Anti-CD3 monoclonal antibody	NasVax (Israel)	Phase II	T-cell surface glycoprotein CD3 molecule targeting agent Induces regulatory T cells and anti-inflammatory immune responses
OCA INT-747	Dainippon Sumitomo (Japan) Intercept (USA)	Phase II	FXR ligand
GFT-505	GENFIT (France)	Phase II	PPAR α/δ agonist
EPA	Mochida (Japan)	Phase II	Reduces TNF-α expression/improves insulin sensitivity
DR cysteamine	Raptor (USA)	Phase II	A precursor of the potent liver anti-oxidant GSH
Mitoquinone	Antipodean	Phase II	Mitochondria-targeted anti-oxidant
BLX-1002	BEXEL Pharmaceuticals	Phase II	IL-1β ligand inhibitor/insulin sensitizer/anti-inflammatory
Oltipraz	PharmaKing (South Korea)	Phase II	Inhibitor of AMPK-S6K1 pathway and LXRα-SREBP-1c pathway

AMPK: Adenosine monophosphate-activated protein kinase; DR cysteamine: Delayed-release cysteamine; EPA: Eicosapentaenoic acid; FXR: Farnesoid X receptor; GSH: Glutathione; LXR: Liver X receptor; OCA: Obeticholic acid; PPAR: Peroxisome proliferator-activated receptor; SREBP: Sterol regulatory element-binding protein.

currently requires a liver biopsy, which is invasive, somewhat painful and may be associated with life-threatening complications in some individuals [15]. Therefore, non-invasive methods have been proposed for assessing disease severity, such as serum markers and scoring systems (including the Fibro Test [49], the NAFLD fibrosis score [16], the BARD score [50] and the FIB-4 index [51]). The most anticipated serum marker is cytokeratin-18 (CK-18). Plasma CK-18 fragment levels are correlated with the magnitude of hepatocyte apoptosis, and the plasma CK-18 fragment levels were reportedly capable of predicting histological NASH and the severity of disease in a multicenter validation study [52].

Whereas several drugs have been shown to be effective in clinical trials of varying designs, there are currently no approved therapies for NAFLD/NASH, as previously mentioned above. Thus, because of the lack of such modalities, the current research goal for NASH treatment is to develop therapeutic approaches that are widely applicable and that can be used safely for long-term therapy.

6. Scientific rationale

The pathogenesis of NAFLD and NASH has not been fully elucidated. Patients with NAFLD often have atherogenic dyslipidemia (i.e., elevated levels of very low-density lipoprotein (VLDL) and decreased levels of high-density lipoprotein (HDL), and small dense low-density lipoprotein (LDL)). In addition, insulin resistance can lead to an imbalance in lipid metabolism, resulting in increased VLDL secretion. Steatosis results from a discrepancy between triglyceride gain and elimination. A 'two-hit' model describing NAFLD progression to NASH suggests that lipid accumulation in hepatocytes because of insulin resistance is followed by hepatocellular injury caused by inflammation and oxidation [53]. Pro-inflammatory cytokines, oxidative stress and factors outside the liver can facilitate the conversion from hepatic steatosis to NASH.

Insulin resistance, which is frequently seen in obese individuals, is tightly linked to this process, as it alters nutrient distribution among tissues and nutrient metabolism. Impaired insulin signaling leads to enhanced adipose tissue lipolysis and an increased flow of free fatty acids to the liver, contributing to lipid peroxidation and the formation of ROS [54]. Inflammation is triggered by signals derived from adipocytes (e.g., cytokines such as TNF-α and IL-6), immune cells (e.g., macrophages, and Kupffer cells) and nutrients (e.g., ω-6 fatty acids) as well as intestinal microbacteria (e.g., endotoxins). To date, therapeutic trials for NASH have had the reduction of steatosis, insulin resistance, oxidative stress, inflammation and even fibrosis as treatment goals.

7. Competitive environment

Clinical trials examining new therapeutic drugs for NASH that act via various mechanisms are being performed in several countries, and these drugs may strongly influence current NASH treatment. The emerging drugs for NASH are mostly in Phase I or Phase II of drug development, although few drugs are in Phase III or Phase IV. Some of these drugs, especially those currently in Phase II of drug development, are summarized in Table 2.

7.1 Oral anti-CD3 monoclonal antibody

Intravenous anti-CD3 antibody immunotherapy has been approved for > 20 years for the treatment of graft rejection after transplantation. However, its use for chronic inflammatory and autoimmune disease indications has been avoided because of significant adverse events after injection. Preclinical studies have shown that an oral anti-CD3 monoclonal antibody does not induce side effects and is effective for preventing the induction or progression of disease in a range of animal models

for inflammatory and autoimmune diseases [55]. These studies have also shown that oral anti-CD3 monoclonal antibody induces regulatory T cells and an anti-inflammatory immune response responsible for the efficacy of the drug.

A Phase IIa clinical trial in 36 patients with NASH showed that oral anti-CD3 immunotherapy was safe and well tolerated and that it induced positive trends in clinical biomarkers and immunological markers in groups receiving oral anti-CD3 monoclonal antibody, but not in a placebo group. Some of these trends were statistically significant despite the very small group sizes [56].

7.2 Obeticholic acid

Obeticholic acid (OCA, also known as INT-747) is a 6α-ethyl derivative of chenodeoxycholic acid (CDCA) and is a first-in-class selective farnesoid X receptor (FXR) agonist that was originally described for its anticholestatic and potentially broader hepatoprotective properties [57]. FXR is a member of the nuclear receptor superfamily and is mainly expressed in the liver, intestine, kidney and, to a lower extent, adipose tissue. The activation of FXR inhibits bile acid synthesis from cholesterol and also protects against the toxic accumulation of bile acids through increased conjugation in the liver and secretion into bile canaliculi [58].

Preclinical studies have shown the capacity of OCA to increase insulin sensitivity and to regulate glucose metabolism, to modulate lipid metabolism, to protect hepatocytes against bile acid-induced cytotoxicity and to exert anti-inflammatory properties along with marked antifibrotic effects [59].

OCA has been evaluated in a Phase II clinical trial for patients with NAFLD who have type 2 diabetes. This trial was a multicenter, placebo-controlled study that evaluated two oral daily doses of OCA: 25 and 50 mg for 6 weeks. In this short study, the level of a liver fibrosis marker and the transaminase level decreased significantly in the group that received 25 mg/day, compared with a placebo [60]. Based on this study with a limited number of patients, larger multicenter Phase II studies of OCA in NASH patients for 72 weeks are now currently ongoing in the USA [61] and Japan [62].

7.3 GFT-505

PPARs are fatty acid-activated nuclear receptors that regulate an array of physiological processes [63]. The PPAR nuclear receptor subfamily is composed of three members: PPARα, PPARγ and PPARδ. Agonists of two of these receptors are currently used therapeutically, with the hypolipidemic fibrate drugs acting as PPARα agonists and the insulin-sensitizing TZDs acting as PPARγ agonists. Currently, there are no clinically used drugs that target PPARδ. PPARδ is widely expressed and plays a critical role in mitochondrial function, muscle development, fatty acid oxidation and insulin sensitivity [64].

GFT-505 is a PPAR modulator with preferential activity on PPARα and additional activity on PPARδ. Phase I studies in healthy volunteers have shown that GFT-505 induces a dose-dependent reduction in plasma triglyceride and an increase in HDL-cholesterol levels [65]. Furthermore, two Phase IIa studies have provided evidence that GFT-505 improved multiple metabolic parameters in abdominally obese patients with either combined dyslipidemia or prediabetes [66]. In these studies, GFT-505 improved not only glucose and lipid metabolism, but also liver dysfunction. These results position this dual PPARα/δ agonist as a new drug candidate for NASH. A Phase IIb study to evaluate the efficacy and safety of GFT-505 80 and 120 mg once daily for 52 weeks in patients with NASH is now ongoing [67].

7.4 Eicosapentaenoic acid

Evidence from epidemiologic studies and randomized controlled trials has shown that supplementation with *n*-3 polyunsaturated fatty acids (n-3 PUFA), which are abundantly present in fish oil, lowers blood triglyceride levels and reduces the risk of coronary heart disease, sudden death and mortality [68]. Eicosapentaenoic acid (EPA) is one of the principal components of n-3 PUFA, and highly purified EPA is widely used for treatment of hyperlipidemia and atherosclerosis. Treatment with n-3 PUFA, a mixture of EPA and docosahexaenoic acid, reportedly ameliorates hepatic steatosis and necroinflammation in humans [69] and rats [70], probably because of a reduction in hepatic TNF-α expression and the improvement of insulin sensitivity.

A pilot trial evaluated the efficacy and safety of EPA in 23 biopsy-proven NASH patients [71]. In this study, highly purified EPA (2700 mg/day) was administered for 12 months, and 87% of the patients who enrolled experienced a decrease in serum ALT levels, which normalized in 22% of the cases. Similarly, an improvement in hepatic steatosis was documented using hepatic ultrasonography in 74 patients. Eighty-six percent of the seven patients who underwent serial biopsies showed an improvement in several key features of NASH, including hepatic steatosis, fibrosis, lobular inflammation and hepatocyte ballooning, and 43% demonstrated a significant improvement in their NAS of > 3 points. This beneficial effect of EPA was thought to be attributable to its anti-inflammatory and anti-oxidative properties. To confirm these results, a Phase II double-blind, placebo-controlled trial has recently been completed [72], the detailed results of which are eagerly awaited.

7.5 Delayed-release cysteamine (DR cysteamine, RP103)

One of the major contributory factors in the progression of NAFLD to NASH is increased oxidative stress caused by either the excessive production of ROS or a decreased antioxidant defense. The most abundant intracellular antioxidant agent is the free thiol tripeptide glutathione (GSH). GSH depletion contributes to hepatocellular injury and fibrosis [73]. Exogenous GSH does not enter cells readily, but drugs such as *N*-acetylcysteine and cysteamine are able to do so; the repletion of GSH in this manner can be effective for the

treatment of conditions where GSH depletion occurs (such as acetaminophen toxicity).

Cysteamine can act by increasing the cellular thiol pool, scavenging reactive oxygen intermediates such as superoxide-free radicals and hydrogen peroxide, decreasing lipoperoxidation and GSH peroxidase activity and increasing GSH production [74]. RP103 is an enteric-coated, microbead formulation of cysteamine bitartrate [75]. This drug is administered as an enteric-coated tablet to reduce the dosing frequency and associated upper gastrointestinal side effects. In a pilot study, enteric-coated cysteamine reportedly reduced the ALT and AST levels in children with NAFLD without reducing the body mass index [76]. A Phase IIb study of RP103 with an estimated enrollment of 160 pediatric patients with biopsy-proven moderate to severe NAFLD began in June 2012 [77].

7.6 Mitoquinone

Mitoquinone is a mitochondria-targeted anti-oxidant that decreases mitochondrial oxidative damage. This novel class of compounds combines a potent anti-oxidant, such as the ubiquinone moiety of mitoquinone, with a lipophilic triphenylphosphonium cation, and the compound's large mitochondrial membrane potential allows the anti-oxidant to accumulate within mitochondria by several 100-fold after oral administration [78]. A Phase II study has demonstrated that mitoquinone significantly decreased the plasma ALT and AST levels in patients with chronic hepatitis C who were not responsive to pegylated IFN plus ribavirin therapy [79]. Strong evidence indicates that mitochondrial oxidative stress contributes to many other chronic liver diseases, including NASH [80]. A Phase II, double-blind randomized placebo-controlled trial in patients with NAFLD was designed, but this study was ultimately terminated due to poor participant recruitment [81].

7.7 BLX-1002

BLX-1002 is a novel tyrosine-coupled TZD that does not structurally resemble any previously described TZDs. Like other TZDs, BLX-1002 ameliorates hyperglycemia in rodent models of diabetes, but it neither shows a relevant affinity to PPARγ nor does it induce weight gain, as is typically associated with PPARγ-mediated adipogenesis [82]. The search for PPARγ-independent mechanisms of action has so far revealed that BLX-1002 reportedly potentiates glucose-stimulated insulin secretion from pancreatic islet cells [83]. Animal models of NAFLD treated with BLX-1002 reportedly showed a substantial reduction in body weight, plasma glucose and hepatic steatosis, compared with a disease control [84]. BLX-1002 is being examined in a Phase IIa clinical trial for the treatment of NAFLD/NASH in Malaysia.

7.8 Oltipraz

Dithiolethiones, a novel class of adenosine monophosphate-activated protein kinase (AMPK) activators, prevent insulin resistance through AMPK-dependent p70 ribosomal S6 kinase-1 (S6K1) inhibition. It is reported that the modulation of S6K1 by oltipraz inhibited the development of insulin resistance and hyperglycemia through the AMPK-S6K1 pathway [85]. Also, some research has reported that liver X receptor (LXR)-α-mediated increases in sterol regulatory element-binding protein (SREBP)-1c promote the expression of lipogenic genes and enhance fatty acid synthesis and oltipraz inhibits LXR-α and SREBP-1c [86]. Therefore, oltipraz is considered to inhibit fatty acid synthesis by acting on the AMPK-S6K1 and LXRα-SREBP-1c pathways in the liver. Oltipraz is currently being examined in a Phase II trial for NAFLD in South Korea [87].

8. Potential development issues

In addition to Phase II drugs, which were described in the previous section, several drugs that could be candidates for future large-scale examinations have recently been reported (Table 3). At present, these drugs typically improve one element of metabolic syndrome, and most of them are not specific for NAFLD and have not been evaluated in NAFLD/NASH patients.

Ipragliflozin (ASP-1941) is a novel sodium glucose co-transporter 2 (SGLT2) inhibitor that is in clinical development for the treatment of type 2 diabetes mellitus. Ipragliflozin has been shown to increase urinary glucose excretion and to reduce hyperglycemia and body weight in patients with type 2 diabetes mellitus. Ipragliflozin reduced the hepatic triglyceride content in diabetic obese mice and prevented hepatic triglyceride accumulation and fibrosis in rats fed a choline-deficient and amino acid-defined (CDAA) diet, while it prevented inflammation and fibrosis in rats fed a methionine- and choline-deficient (MCD) diet [88]. These positive findings support future clinical studies in patients with NASH.

Anti-angiogenic treatment has proven to be an effective treatment for several chronic liver diseases such as cirrhosis and hepatocellular carcinoma. Preclinical data for an anti-αVEGFR2 antibody have been presented as a potential treatment for NASH. The inhibition of VEGFR2 has been shown to protect against the development of steatosis and inflammation in a diet-induced mouse model for NASH [89]. This finding warrants further investigation of the role of angiogenesis and lipogenesis in the pathophysiology of NASH.

Galactin-3, a galactose binding protein, was recently identified as a potential target for drug therapy since galactin-3 null mice are resistant to the development of NASH. GR-MD-02 is a complex carbohydrate drug that binds galectin-3. Treatment with this galectin-3 targeting drug improved steatosis, ballooning and inflammation and reversed fibrosis in a mouse model of NASH [90].

ISIS-DGAT2Rx inhibits diacylglycerol acyltransferase-2 (DGAT-2), a key component in the synthesis of triglycerides. By reducing DGAT-2, ISIS-DGAT2Rx is expected to reduce liver fat in patients with NASH [91].

Table 3. List of some NASH drugs currently in the preclinical stage of development.

Compound	Company	Stage of development	Mechanism of action
Ipraglifozin (ASP-1941)	Kotobuki Pharmaceutical (Japan)	Preclinical	SGLT2 inhibitor
Anti-VEGFR2 antibody	ThromboGenics NV (Belgium)	Preclinical	Anti-inflammatory; VEGFR2 antagonist
GR-MD-02	Galectin Therapeutics (USA)	Preparation for clinical	Galecton-3 inhibitor
ISIS-DGAT2Rx	ISIS Pharmaceuticals (USA)	Preclinical	DGAT-2 inhibitor
PYN22	Phynova Group (UK)	Preclinical	A highly purified Chinese plant extract Antihyperlipidemic agent
Nanoveson	ALP Life Sciences (USA)	Preclinical	Triggers liver triglyceride remodeling into bile phospholipids and lipid membrane fusion and aggregation in the intestines
Sitagliptin	Merck & Co. (USA)	Preclinical	DPP-4 inhibitor

DGAT-2: Diacylglycerol acyltransferase-2; DPP-4: Dipeptidyl peptidase-4; SGLT2: Sodium glucose co-transporter 2.

PYN22 is a highly purified Chinese plant extract that has been selected as a candidate for the treatment of NAFLD/NASH, based on preclinical work in both China and the UK that suggests that PYN22 may have a direct effect on body weight and fat levels in the liver and blood [92]. The mechanisms of action of PYN22 have not yet been fully elucidated, but studies have shown that PYN22 effectively lowers the expression of several key genes associated with obesity and fatty liver diseases, such as those affecting lipogenesis, adipogenesis, fatty acid oxidation, insulin sensitivity, adiponectin expression and acetyl-CoA carboxylase expression [93].

Nanoveson triggers liver triglyceride remodeling into bile phospholipids and lipid membrane fusion and aggregation in the intestines [94]. A clinical trial of this drug for the treatment of NAFLD/NASH is expected to occur in the near future.

Sitagliptin is a dipeptidyl peptidase-4 (DPP-4) inhibitor that has been approved as an adjunct to diet and exercise for improving glycemic control in adults with type 2 diabetes mellitus. In a short-term study for NAFLD patients with type 2 diabetes mellitus, sitagliptin improved not only the parameters of diabetes, but also the levels of transaminases [95]. Furthermore, this study suggested that sitagliptin might have the potential to influence the improvement of steatosis. A large-scale clinical trial is warranted in the future.

9. Conclusion

Currently, there is a lack of consensus regarding the most effective and appropriate pharmacologic therapy for NASH. Numerous clinical trials have been designed and performed in many countries, as described in this review. Existing treatments have been assessed in detail and have been prescribed for patients with NASH, but they lack sufficient power to show a definitive benefit. The NASH market is growing with the increasing incidence and prevalence of this disease, and this market also poses huge unmet needs. Many companies have worked hard and competitively on the development of new therapeutic drugs for NASH, with the majority of potential drugs having reached the Phase II stage of development. Further investigations for safer and more effective pharmacologic therapies are needed.

10. Expert opinion

As mentioned repeatedly in this review, a definite treatment for NASH remains to be defined despite strong efforts and numerous clinical trials. This is a major weakness of research on pharmacologic therapy for NASH, and overcoming this situation is the most important mission for current researchers.

The field of NASH is evolving rapidly, and many advances have been made in the understanding of the pathogenesis of NASH. Recently, the progression of NASH has been accepted to be complex, involving multiple genetic factors interacting with the environment and lifestyle, since only a portion of NAFLD patients develops NASH. The patatin-like phospholipase 3 (PNPLA3) gene has been identified as a major determinant for the development of NAFLD, and it was shown to be associated with not only fatty liver and triglycerides content, but also inflammation and fibrosis [96-98]. However, it will take many years for this knowledge to be applied to the treatment of NASH, such as the widespread use of genomic screening to identify patients with a genetic predisposition to NASH.

The excessive mortality among patients with NASH is associated with liver-related deaths, CVD and non-hepatocellular cancers [99]. An ideal treatment for NASH should improve not only liver disease, but should also reduce the risks of adverse cardiovascular outcomes and the development of diabetes and cancers. However, this goal is likely to be too high in the context of clinical trials designed to obtain approval for the use of pharmacological agents capable of improving liver disease, as several decades are likely to be needed to achieve

this goal. Numerous clinical trials have been designed and performed in many countries, and the only way to achieve this ultimate goal is to accumulate the results of these relatively short-term clinical trials.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

The association of insomnia with gastroesophageal reflux symptoms in biopsy-proven nonalcoholic fatty liver disease

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Abstract
Background It is suggested that nonalcoholic fatty liver disease (NAFLD), including nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH), can be associated with insomnia and gastro-esophageal reflux disease (GERD). The relationship between GERD and insomnia in subjects with biopsy-proven NAFLD was investigated.
Methods This study enrolled 123 patients with biopsy-proven NAFLD. Insomnia was assessed by the Athens Insomnia Scale (AIS), a self-assessment psychometric instrument designed to quantify sleep difficulty based on ICD-10 criteria; AIS scores ≥ 6 were considered positive for insomnia. GERD symptoms were evaluated using a frequency scale for the symptoms of GERD (FSSG); FSSG scores ≥ 8 were considered positive. Logistic regression models were used to evaluate the association of insomnia with GERD, after adjusting for potential confounders. Thirteen patients with GERD were treated with the proton pump inhibitor rabeprazole (RPZ; 10 mg/day), for 12 weeks.
Results Of the 123 patients, 76 (62 %) were female and 87 (71 %) were obese, with 34 (28 %) having AIS scores ≥ 6 and 31 (25 %) having FSSG scores ≥ 8. Liver biopsy revealed that 40 patients (33 %) had NAFL and 83 (67 %) had NASH. FSSG and AIS scores were similar in the two groups. HOMA-IR, FSSG scores and γGT (GGT) concentrations were significantly higher in insomniacs than in non-insomniacs. Logistic regression analysis demonstrated that FSSG score and GGT concentration were independently associated with insomnia. RPZ treatment resulted in significantly reductions in both AIS and FSSG scores.
Conclusions Nearly 30 % of patients with biopsy-proven NAFLD had insomnia, which was related to GGT and GERD and could be relieved by RPZ treatment.

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Abbreviations

BMI	Body mass index
GERD	Gastro-esophageal reflux disease
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis

Introduction

Nonalcoholic fatty liver disease (NAFLD) [1] is the most common chronic liver disease in many developed countries and results in serious public health problems worldwide. NAFLD includes a wide spectrum of liver diseases, ranging from nonalcoholic fatty liver (NAFL), which is usually benign, to nonalcoholic steatohepatitis (NASH), which may progress to liver cirrhosis (LC), hepatic failure and hepatocellular carcinoma (HCC) in the absence of significant alcohol consumption [2]. A large proportion of NAFLD patients are asymptomatic, but some occasionally experience fatigue, anxiety, and/or insomnia, resulting in a significant decrement in quality of life (QOL) [3]. In middle-aged Koreans, short sleep duration and poor sleep quality were found to be significantly associated with an increased risk of NAFLD [4]. Similarly, short sleep duration was associated with NAFLD in the general Japanese population [5]. However, the mechanisms underlying the association between insomnia and NAFLD remain unknown. Sleep is important to maintain body homeostasis, with sleep problems associated with all-cause mortality [6].

In addition to being associated with sleep problems, NAFLD was found, in two recent studies from Japan and Italy, to be associated with a high prevalence of the symptoms of gastro-esophageal reflux disease (GERD) [7, 8]. Evidence has emerged suggesting a link between metabolic syndrome, specifically obesity and visceral fat accumulation, and the onset of GERD. Studies throughout the world have shown that GERD is associated with sleep problems [9–14]. For example, a population-based study from Sweden showed positive associations among the presence of insomnia, sleeplessness, problems falling asleep, and risk of GERD [10]. In addition, an analysis of 19864 healthy adults in Japan found that poor sleep quality and irregular dietary habits were strong risk factors for high scores on the frequency scale for the symptoms of GERD (FSSG) [15]. Thus, it can be hypothesized that GERD symptoms may be responsible for insomnia in patients with NAFLD. To our knowledge, no study to date has assessed the prevalence of insomnia or GERD, or their association, in patients with biopsy-proven NAFLD.

Rabeprazole (RPZ), a proton pump inhibitor (PPI), is a potent and irreversible inhibitor of the H(+)/K(+)-ATPase gastric pump and is indicated for the treatment of GERD, Zollinger–Ellison syndrome, and duodenal and gastric ulcers. Moreover, the combination of RPZ and antibiotics is indicated for the eradication of *Helicobacter pylori*. RPZ is therefore expected to be effective in the treatment of GERD patients with sleep disturbances [16, 17]. This study was designed to evaluate the prevalence of insomnia and GERD in patients with biopsy-proven NAFLD; to compare the rates of insomnia and GERD in patients with NASH and NAFL; to determine independent predictors of insomnia, including FSSG score, among these patients; and to evaluate the effect of RPZ on insomnia.

Methods

Study population

The study included a total of 123 patients with well-characterized, liver biopsy–confirmed NAFLD who completed the FSSG questionnaire assessing symptoms of GERD and the Athens Insomnia Scale (AIS) questionnaire. All patients underwent biopsies at one of the seven hepatology centers included in the Japan Study Group of NAFLD (JSG-NAFLD): Center for Digestive and Liver Diseases, Nara City Hospital; Division of Gastroenterology, Yokohama City University Graduate School of Medicine; Department of Medicine and Molecular Science, Graduate School of Biomedical Sciences, Hiroshima University; Department of Gastroenterology and Hepatology, Kochi Medical School; Department of Internal Medicine, Saga Medical School, Saga University; Department of Hepatology, Graduate School of Medicine, Osaka City University; and the Department of Gastroenterology and Hepatology, Kyoto Prefectural University of Medicine.

NAFLD was diagnosed based on liver biopsy findings of steatosis in ≥ 5 % of hepatocytes and the exclusion of other liver diseases, including viral hepatitis, autoimmune hepatitis, drug-induced liver disease, primary biliary cirrhosis, biliary obstruction, hemochromatosis, Wilson's disease, and α-1-antitrypsin-deficiency-associated liver disease. Patients consuming more than 20 g of alcohol per day, those with evidence of decompensated LC or HCC, those with psychiatric disorders or psychiatric drug users, and those taking PPIs and/or histamine H2-receptor antagonists were excluded. All patients provided written informed consent at the time of liver biopsy, and the study was conducted in conformance with the Declaration of Helsinki.

Laboratory and clinical parameters

Venous blood samples were taken in the morning after a 12-h overnight fast. Laboratory assays included blood cell counts and measurements of serum concentrations of aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), cholinesterase (ChE), total cholesterol, triglycerides, fasting plasma glucose (FPG), immunoreactive insulin (IRI), ferritin, hyaluronic acid, and type IV collagen 7S. These parameters were measured using the standard techniques of clinical chemistry laboratories.

Body mass index (BMI) was calculated as weight in kilograms/(height in meters)^2, with obesity defined as a BMI > 25 kg/m^2, according to the criteria of the Japan Society for the Study of Obesity [18]. Patients taking oral hypoglycemic medication, and those with a random glucose concentration > 200 mg/dl or a fasting glucose concentration > 126 mg/dl, were regarded as positive for hyperglycemia [19]. Patients with serum cholesterol concentrations > 220 mg/dl or triglyceride concentrations > 160 mg/dl were diagnosed with dyslipidemia. Patients taking antihypertensive agents and those having a resting recumbent blood pressure $\geq 140/90$ mmHg on at least two occasions were regarded as having hypertension [20].

GERD score

The FSSG is a questionnaire widely used to diagnose GERD [21–24] and to evaluate the effectiveness of any treatment [21, 25].The FSSG consisted of 12 questions assessing the frequency of symptoms (never, 0; occasionally, 1; sometimes, 2; often, 3; and always, 4). Patients with FSSG scores ≥ 8 were considered positive for GERD; at this cut-off point, the FSSG had a sensitivity of 62 %, a specificity of 59 %, and an accuracy of 60 % in assessing GERD [21].

Insomnia scale

The intensity of sleep difficulty was evaluated using the AIS, a self-administered psychometric tool with high consistency, reliability and external validity (Table 1) [26, 27]. The AIS consists of eight items, five of which are used to assess insomnia, and the three used to assess well-being, functional capacity, and sleepiness during the day. The full eight-item version (AIS-8) was developed for clinical settings, while the five-item version (AIS-5) can be used to assess sleep quantity and quality. These first five questions (AIS-5) are used to assess difficulty with sleep induction, awakenings during the night, early morning awakening, total sleep time and overall quality of sleep. The last three items in the AIS-8 refer to

Table 1 Athens Insomnia Scale (AIS) [26]

Sleep induction (time it takes you to fall asleep after turning-off the lights)			
0: No problem	1: Slightly delayed	2: Markedly delayed	3: Very delayed or did not sleep at all
Awakening during the night			
0: No problem	1: Minor problem	2: Considerable problem	3: Serious problem or did not sleep at all
Final awakening earlier than desired			
0: Not earlier	1: A little earlier	2: Markedly earlier	3: Much earlier or did not sleep at all
Total sleep duration			
0: Sufficient	1: Slightly insufficient	2: Markedly insufficient	3: Very insufficient or did not sleep at all
Overall quality of sleep (no matter how long you slept)			
0: Satisfactory	1: Slightly unsatisfactory	2: Markedly unsatisfactory	3: Very unsatisfactory or did not sleep at all
Sense of well-being during the day			
0: Normal	1: Slightly decreased	2: Markedly decreased	3: Very decreased
Functioning (physical and mental) during the day			
0: Normal	1: Slightly decreased	2: Markedly decreased	3: Very decreased
Sleepiness during the day			
0: None	1: Mild	2: Considerable	3: Intense

Instructions this scale is intended to record own assessment of any sleep difficulty you might have experienced. Please, check (by circling the appropriate number) the items above to indicate your estimate of any difficulty, provided that it occurred at least three times per week during the last month

The period of the self-assessment may vary, depending on the design of a given study. Whenever the self-assessment pertains to a period other than that of the last month, the second sentence of the instructions should be rephrased accordingly