daytime symptoms that often result from sleep disorders, such as narcolepsy and obstructive sleep apnea, in patients with insomnia. Each item on the AIS was rated from 0 (*no problem at all*) to 3 (*very serious problem*). Total scores can range from 0 to 24, with scores ≥ 6 and < 6 representing the presence and absence of insomnia, respectively. This cutoff point had a sensitivity of 93 %, a specificity of 85 % (90 % overall correct case identification), a positive predictive value (PPV) of 41 % and a negative predictive value (NPV) of 99 % [27].

Responders were asked to calculate their scores if they had experienced sleep difficulties at least three times a week during the previous month.

Liver histology

All enrolled patients underwent a percutaneous liver biopsy under ultrasonic guidance or peritoneoscopy. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson-trichrome, reticulin silver stain, and Perls' Prussian blue. The specimens were evaluated by two hepatic pathologists (S.T. and Y.S.), who were blinded to the clinical findings. An adequate liver biopsy sample was defined as a specimen of length > 1.5 cm and/or having more than 6 portal tracts. NASH was defined as steatosis with lobular inflammation and ballooning degeneration, with or without Mallory–Denk body or fibrosis. Patients with liver biopsy specimens showing simple steatosis or steatosis with nonspecific inflammation were identified as the NAFL cohort [28]. Specimens with steatosis of < 5, 5–33, > 33–66, and > 66 % were scored as having steatosis grades of 0, 1, 2, and 3, respectively [29]. Histological grade and stage were scored as described [30]. Necroinflammatory grades of 1, 2, and 3, were defined as mild, moderate and severe hepatocellular steatosis,

Table 2 Clinical characteristics of enrolled patients with NAFL and NASH

Clinical parameter	Total (n = 123 [100 %])	NAFL (n = 40 [33 %])	NASH (n = 83 [67 %])	P value
Age (years)	59 (14–82)	56 (20–78)	62 (14–82)	0.0025
Gender (female)	76 (62 %)	17 (43 %)	59 (71 %)	0.0030
BMI (kg/m^2)	26.6 (16.6–43.4)	26.6 (18.9–43.4)	27.3 (16.6–41.0)	0.1405
Obesity (BMI > 25)	87 (71 %)	27 (68 %)	60 (72 %)	0.6731
Dyslipidemia (yes [%])	46 (37 %)	12 (30 %)	34 (41 %)	0.3202
Hypertension (yes [%])	48 (39 %)	10 (25 %)	38 (46 %)	0.0310
Type 2 diabetes (yes [%])	55 (45 %)	13 (33 %)	42 (51 %)	0.0811
Hemoglobin (g/dl)	14.1 (10.5–18.3)	14.8 (10.6–18.3)	13.8 (10.5–16.7)	0.0728
Platelet count ($\times 10^4$/μl)	21.4 (4.6–78.5)	23.5 (13.0–78.5)	20.8 (4.6–45.4)	0.0125
AST (IU/l)	45 (17–186)	37 (17–151)	51 (18–186)	0.0001
ALT (IU/l)	69 (12–358)	61 (15–358)	71 (12–218)	0.2425
GGT (IU/l)	61 (20–391)	60 (20–319)	62 (21–391)	0.6382
Cholinesterase (IU/l)	371 (167–547)	378 (266–545)	370 (167–547)	0.2873
Total cholesterol (mg/dl)	209 (87–335)	218 (127–335)	203 (87–319)	0.0183
Triglyceride (mg/dl)	156 (61–659)	155 (61–416)	162 (66–659)	0.4849
HDL-C (mg/dl)	50 (23–290)	49 (31–77)	52 (23–290)	0.7727
Ferritin (ng/ml)	163 (5–1100)	113 (10–1100)	210 (5–923)	0.0160
FPG (mg/dl)	96 (60–452)	96 (60–161)	96 (60–452)	0.3571
IRI (μU/ml)	11.4 (1.59–49.5)	8.4 (1.6–46)	13.2 (2.8–49.5)	<0.0001
HOMA-IR	2.62 (0.38–33.04)	1.87 (0.38–13.63)	3.02 (0.65–33.04)	<0.0001
Hyaluronic acid (ng/ml)	37 (9–3480)	22 (9–149)	49 (9–3480)	0.0001
Type IV collagen 7S (ng/ml)	4.5 (2.7–13)	3.7 (2.8–7.1)	5.1 (2.7–13.0)	<0.0001
FSSG	4 (0–38)	4 (0–29)	3 (0–38)	0.5009
FSSG ≥ 8 (n [%])	31 [25 %]	10 [25 %]	21 [25 %]	1.0000
AIS	3 (0–15)	3 (0–12)	4 (0–15)	0.5591
AIS ≥ 6 (n [%])	34 [28 %]	10 [25 %]	24 [29 %]	0.8299

Results are presented as numbers with percentages in parenthesis for qualitative data or as mean ± SD for quantitative data

BMI body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, *IRI* immuno-reactive insulin

P values were calculated by t test or χ^2 analysis

ballooning and inflammation (acinar and portal), respectively. The severity of hepatic fibrosis (stage) was scored as: stage 1, zone 3 perisinusoidal fibrosis; stage 2, zone 3 perisinusoidal fibrosis with portal fibrosis; stage 3, zone 3 perisinusoidal fibrosis and portal fibrosis with bridging fibrosis; and stage 4, cirrhosis.

Treatment with RPZ

Thirteen NAFLD patients with GERD symptoms (11 females and 2 males) were administered 10 mg/day RPZ for 12 weeks. These patients completed both the FSSG and AIS before and after RPZ treatment.

Statistical analysis

Quantitative results are presented as medians and ranges, and qualitative results as numbers and percentages. Statistical differences in quantitative data were determined using the Mann–Whitney U test or Wilcoxon rank-sum test, and differences in qualitative data using Fisher's exact probability test or χ^2 analysis (Tables 2, 4, 5; Figs. 1, 2, 3, 4). Correlations were calculated by Spearman rank correlation analysis (Table 3). Multivariate logistic regression analysis was used to identify variables independently associated with the occurrence of insomnia (Table 6). Statistical significance was defined as a P value < 0.05.

Results

Characteristics of study subjects

Table 2 summarizes the clinical and laboratory data of the patient population. Of the 123 patients with NAFLD, 76 (62 %) were female, and 87 (71 %) were obese (BMI > 25 kg/m^2). Histologically, 83 patients (67 %) were diagnosed with NASH, and 40 (33 %) with NAFL. Patients with NASH were significantly older; were more predominantly female; were more likely to have hypertension and type 2 diabetes; had lower platelet counts and total cholesterol concentrations; and had higher levels of AST, ferritin, IRI, HOMA-IR, hyaluronic acid, and type IV collagen 7S. Of the 83 patients with NASH, 41 (49 %), 22 (27 %), 13 (16 %), and 7 (8 %) had stage 0–1, 2, 3, and 4 fibrosis, respectively.

Comparisons between NASH and NAFL

The distribution of AIS scores in patients with NAFL and NASH is shown in Fig. 1. Overall, 34 of the 123 patients (28 %) with NAFLD had AIS scores ≥ 6, diagnostic of insomnia, including 10 of 40 (25 %) patients with NAFL and 24 of 83 (29 %) with NASH ($P = 0.8299$). Males and females had similar median AIS scores [3 (range 0–13) vs. 3 (range 0–15), $P = 0.7954$] and a similar prevalence of insomnia [26 % (12/47] vs. 29 % (22/76), $P = 0.8359$].

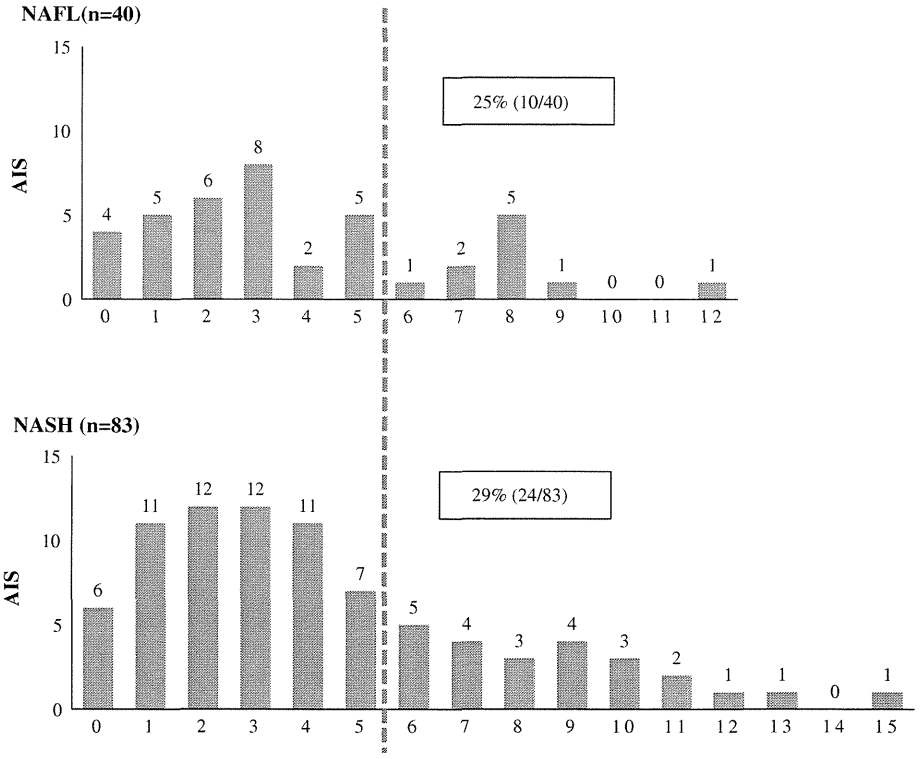

Fig. 1 The distribution of Athens Insomnia scale (AIS)

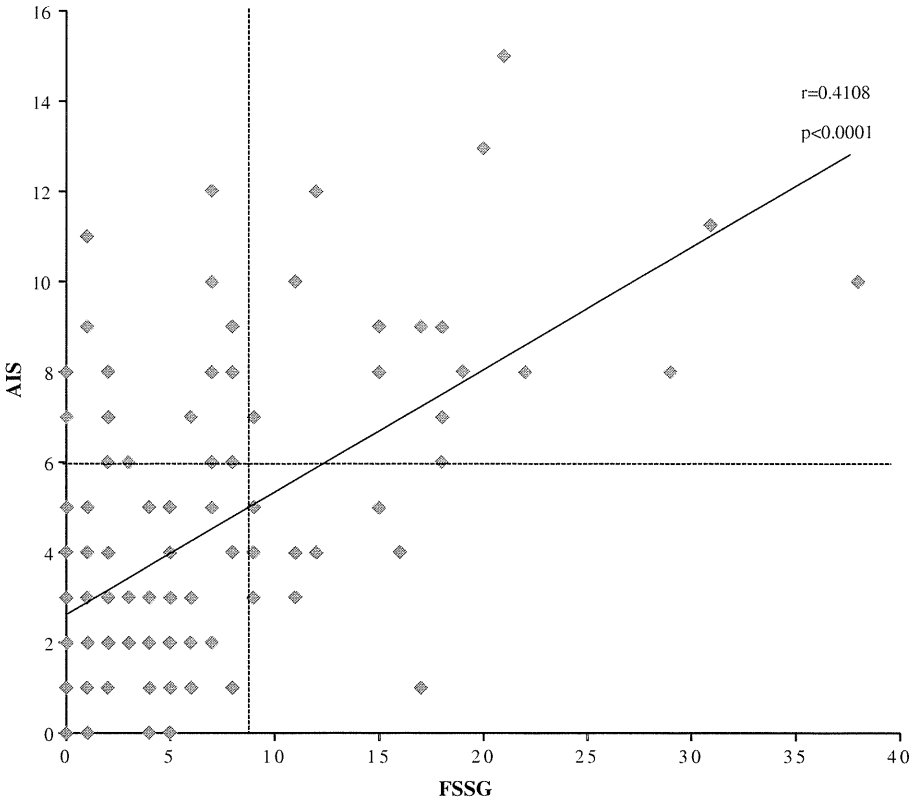

Fig. 2 Correlation between FSSG and AIS. A significant positive correlation was found between AIS and FSSG

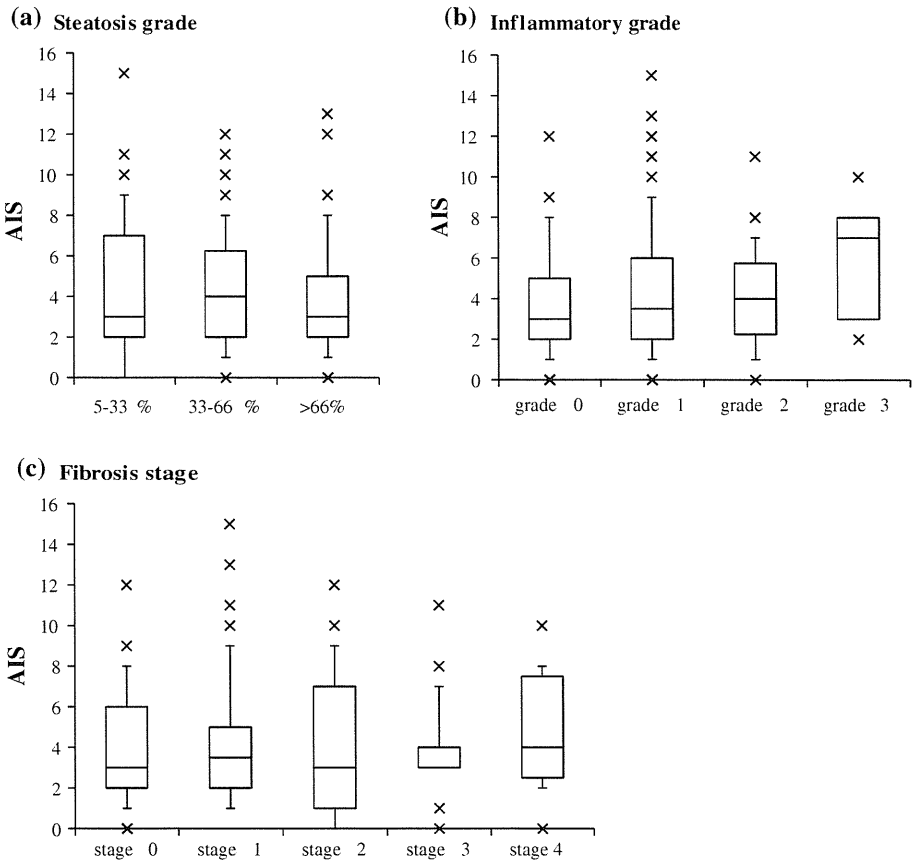

Fig. 3 Relationship between AIS and histological findings. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %

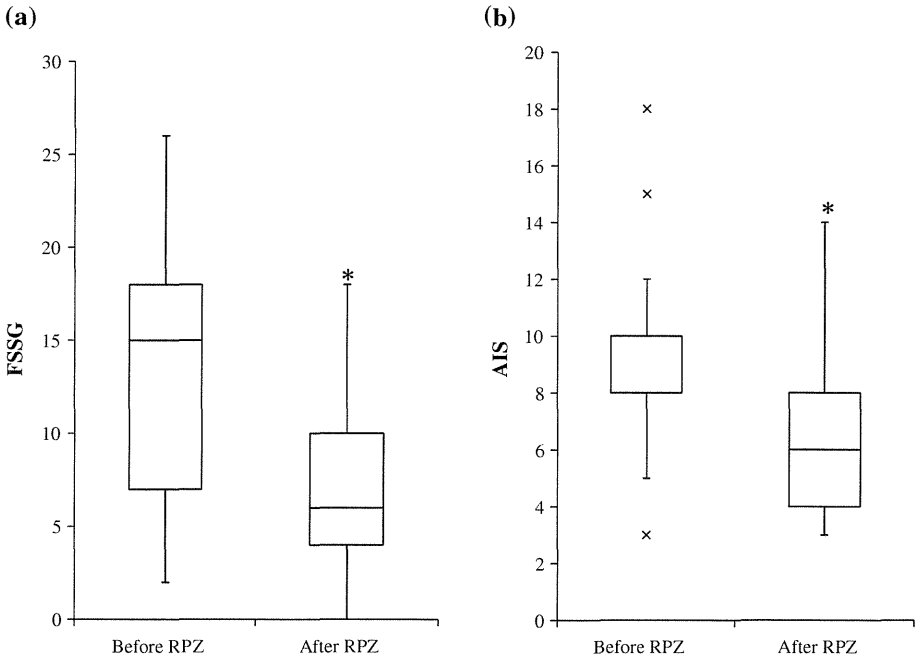

Fig. 4 Effects of rabeprazole (RPZ) on the improvement of GERD symptoms and insomnia. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %. **a** Change in the total FSSG score. RPZ significantly reduced total FSSG scores. $^{*}P = 0.0071$ compared to baseline response before treatment. **b** Change in the total AIS. RPZ significantly reduced total AIS. $^{*}P = 0.0144$ compared to baseline response before treatment

Table 3 Correlation between AIS/FSSG and clinical parameters in 123 patients with biopsy-proven NAFLD

Variables	AIS		FSSG	
	Correlation coefficient	*P* value	Correlation coefficient	*P* value
Age	−0.0431	0.6363	−0.2363	0.0085
BMI	−0.0075	0.9345	0.091	0.3128
Hemoglobin	0.0104	0.9100	0.0328	0.7220
Platelet	0.1079	0.2407	0.2197	0.0159
AST	0.0588	0.5197	0.0015	0.9868
ALT	0.0284	0.7558	0.1148	0.2080
AST/ALT ratio	−0.0046	0.9597	−0.1254	0.1689
γGT	0.1545	0.0935	−0.1272	0.1681
Cholinesterase	0.1366	0.1646	0.1683	0.0861
Prothrombin time	0.0359	0.6998	0.1403	0.1296
Cholesterol	0.1366	0.7233	0.0624	0.5039
Triglyceride	−0.0343	0.7132	0.0506	0.5879
HDL-C	−0.0128	0.8943	−0.1166	0.2230
FPG	0.0442	0.6316	−0.2010	0.0277
IRI	−0.1073	0.2412	−0.1570	0.0855
HOMA-IR	−0.0993	0.2806	−0.1948	0.0330
Ferritin	0.0934	0.3081	−0.0490	0.5932
Hyaluronic acid	−0.0340	0.7088	−0.2246	0.0125
Type IV collagen 7S	−0.0193	0.8323	−0.1277	0.1592
FSSG	0.4108	<0.0001	–	–

P values are based on Spearman's non-parametric correlation analysis

FSSG scored did not differ in NAFL and NASH patients, with the prevalence of GERD being 25 % in each group.

Factors positively correlating with AIS and FSSG scores

AIS score was positively correlated with FSSG score ($r = 0.4108$, $P < 0.001$) (Fig. 2), but not with any other parameter (Table 3). FSSG score was positively correlated with platelet count ($r = 0.2197$, $P = 0.0159$), and negatively correlated with age ($r = -0.2363$, $P = 0.0085$), FPG concentration ($r = -0.2010$, $P = 0.0277$), HOMA-IR score ($r = -0.1948$, $P = 0.0330$) and hyaluronic acid concentration ($r = -0.2246$, $P = 0.0125$). BMI, transaminase activities, lipid profiles, and iron parameters were not correlated with AIS or FSSG score.

Correlation between histological findings and AIS scores

Assessment of histological findings in the 123 patients with NAFLD showed that 53 (43 %), 40 (33 %), and 30 (24 %) had steatosis grades 1, 2, and 3, respectively; 30 (24 %), 70 (57 %), 18 (15 %), and 5 (4 %) had inflammation grades 0, 1, 2, and 3, respectively; and 44 (36 %), 38 (31 %), 21 (17 %), 13 (11 %), and 7 (6 %) had fibrosis grades 0, 1, 2, 3, and 4, respectively. Evaluation of correlations between AIS scores and histological findings showed that AIS score was not correlated with steatosis, inflammation, or fibrosis grade (Fig. 3).

Clinical findings in patients with and without insomnia

Comparisons of clinical and laboratory findings in patients with and without insomnia showed that γGT concentrations and FSSG scores were higher, and the prevalence of hypertension and IRI and HOMA-IR scores were lower, in patients with insomnia (Table 4). Moreover, GERD symptoms were significantly more prevalent in patients with than without insomnia (56 vs. 13 %, $P < 0.0001$).

Drug usage

Drug usage in patients involved in this study was shown in Table 5. Beta-blockers users were more prevalent in patients with GERD compared to those without. The prevalence of other drug users was not different between patients with GERD/insomnia and those without.

Table 4 The comparison between insomniacs and non-insomniacs

Clinical parameter	Insomniacs (n = 34 [28 %])	Non-insomniacs (n = 89 [72 %])	P value
Age (years)	56 (35–74)	60 (14–82)	0.8100
Gender (female)	22 (65 %)	54 (61 %)	0.8359
BMI (kg/m^2)	26.4 (21.9–38.6)	26.6 (16.6–43.4)	0.5028
Obesity (BMI > 25)	25 (74 %)	62 (70 %)	0.8252
Dyslipidemia	21 (62 %)	56 (63 %)	1.0000
Hypertension (yes)	8 (24 %)	40 (45 %)	0.0385
Type 2 diabetes (yes)	16 (47 %)	39 (44 %)	0.8400
Hemoglobin (g/dl)	14.4 (11.0–18.3)	14.0 (10.5–17.1)	0.9072
Platelet count (× 10^4/μl)	22.3 (8.7–33.5)	21.1 (4.6–78.5)	0.7137
AST (IU/l)	47 (20–182)	44 (17–186)	0.4682
ALT (IU/l)	71 (12–358)	69 (15–218)	0.6233
GGT (IU/l)	77 (24–391)	59 (20–268)	0.0063
Cholinesterase (IU/l)	373 (208–547)	371 (167–545)	0.7992
Total cholesterol (mg/dl)	206 (125–314)	214 (87–335)	0.3670
Triglyceride (mg/dl)	146 (68–424)	164 (61–659)	0.3864
Ferritin (ng/ml)	128 (11–1100)	170 (5–923)	0.7315
FPG (mg/dl)	94 (70–452)	97 (60–171)	0.7708
IRI (μU/ml)	9.8 (2.8–11.2)	12.0 (1.6–49.5)	0.0326
HOMA-IR	2.10 (0.65–33.04)	2.96 (0.38–16.34)	0.0335
Hyaluronic acid (ng/ml)	32 (9–392)	37 (9–3480)	0.9774
Type IV collagen 7S (ng/ml)	4.7 (2.7–13.0)	4.4 (2.8–10.0)	0.9054
FSSG	8 (0–38)	3 (0–17)	<0.0001
FSSG ≥ 8 (n [%])	19 [56 %]	12 [13 %]	<0.0001
NASH (n [%])	24 [71 %]	59 [66 %]	0.8299

Results are presented as numbers with percentages in parenthesis for qualitative data or as mean ± SD for quantitative data

BMI body mass index, *AST* aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* gamma glutamyl transpeptidase, *FPG* fasting plasma glucose, *IRI* immuno-reactive insulin

P values were calculated by t test or χ^2 analysis

Table 5 Drug usage

Drug usage	FSSG < 8 ($n = 92$)	FSSG ≥ 8 ($n = 31$)	*P* value	Insomniacs ($n = 34$)	Non-insomniacs ($n = 89$)	*P* value
Use of antihypertensive drugs						
Calcium antagonists	26 [28 %]	10 [32 %]	0.6560	7 [21 %]	29 [33 %]	0.2680
ARBs	4 [4 %]	2 [6 %]	0.6412	2 [6 %]	4 [4 %]	0.6679
Beta-blockers	2 [2 %]	4 [13 %]	0.0348	2 [6 %]	4 [4 %]	0.6679
Use of NSAIDs	10 [11 %]	2 [6 %]	0.7285	1 [3 %]	11 [12 %]	0.1762
Use of anticoagulants	2 [2 %]	0 [0 %]	1.0000	0 [0 %]	2 [2 %]	1.0000
Use of digestive drugs	2 [2 %]	0 [0 %]	1.0000	0 [0 %]	2 [2 %]	1.0000

ARBs angiotensin receptor blockers, *NSAIDs* non-steroidal anti-inflammatory drugs

Table 6 Results of multivariate analysis: independent predictors of insomniacs

Variables	Adjusted (multivariate)		
	OR	95 % CI	*P* value
FSSG	1.2315	1.1221–1.3516	<0.0001
GGT	1.0109	1.0031–1.0189	0.0063
IRI	0.9515	0.8908–1.0164	0.1396
Hypertension	0.5503	0.1823–1.6610	0.2893

OR odds ratio, *CI* confidence interval, *FSSG* frequency scale for the symptoms of GERD, *GGT* gamma glutamyl transpeptidase, *IRI* immuno-reactive insulin

Factors independently predictive of insomnia in patients with NAFLD

Multivariate logistic regression analysis showed that plasma GGT concentration [odds ratio (OR) 1.2315, 95 % confidence interval (CI) 1.1221–1.3516, $P < 0.0001$] and FSSG score (OR 1.0109, 95 % CI 1.0031–1.0189, $P = 0.0063$) were significant independent predictors of insomnia (Table 6). In contrast, IRI and HOMA-IR scores and hypertension were not predictive.

Effects of RPZ on insomnia

RPZ treatment of patients with GERD significantly reduced FSSG and AIS scores (Fig. 4). Of the 9 patients with insomnia treated with RPZ, 4 (44 %) showed resolution of insomnia after treatment.

Discussion

This study of Japanese NAFLD patients demonstrated that (1) 28 % had AIS scores ≥ 6, indicative of insomnia; (2) 25 % had FSSG scores ≥ 8, indicative of GERD; (3) AIS and FSSG scores did not differ significantly in patients with NASH and NAFL; (4) FSSG score was independently associated with AIS score; and (5) insomnia could be relieved after treatment with RPZ.

The precise prevalence of insomnia in NAFLD patients has been unclear. Using the AIS, we found that 28 % of patients with biopsy-proven NAFLD had insomnia. Although sleep dysfunction has been defined as a Pittsburgh Sleep Quality Index (PSQI) score > 5.5 in other studies [17, 31], the AIS is satisfactorily validated, simple to perform, and well accepted based on ICD-10 criteria. The AIS has been used to assess insomnia in the general population in Japan. For example, a study of approximately 3000 individuals found that 21.4 % had experienced insomnia during the previous month [32]. The prevalence of insomnia in our NAFLD patients does not seem to be markedly different from that in the general population. Though this precise reason is unknown, one plausible explanation is that about 20–30 % of the general population is estimated to have NAFLD. We should obtain data from sex- and age-matched non-NAFLD population to clarify whether the prevalence of insomnia in NAFLD is really higher compared to that in the general population. Assessments of employees of two local governments in Japan found that 1382 of 5951 males (23.2 %) and 465 of 1500 females (31.0 %), aged 34–59 years, had insomnia [33, 34]. Assessments of middle-aged women found that 27.5–43.6 % had AIS scores ≥ 6 [35, 36]. Taken together, these findings indicate that the prevalence of insomnia is higher in women than in men, across countries and cultures. In contrast, we observed no differences in AIS scores between men and women with NAFLD. Since the discrepancy between our results and previous studies can be explained by a small number of patients involved in this study, a larger number of patients should be examined in the future. Since Yoshioka et al. [34] showed that the gender difference disappeared after adjustment for paid work and family responsibilities, detailed characteristics of patients should be considered to clarify the gender differences. In Japanese studies in which insomnia was

diagnosed using AIS, factors associated with insomnia included work at visual display terminals for $\geq$ 6 h per day [37], job stress [38, 39], and reduced illumination in the workplace [40]. In this study, however, we did not evaluate these work environmental factors.

The mechanisms by which insomnia arises in patients with NAFLD have never been clarified. We found that AIS scores and the incidence of insomnia were similar in patients with NAFL and NASH. AIS scores did not correlate with any histological findings, such as steatosis, inflammation, and fibrosis scores, indicating that histological severity is not important in the pathogenesis of insomnia in patients with NAFLD. In contrast, many studies have explored the associations between life-style related disorders/obesity and sleep disturbance. Changes in secretion of the hormones cortisol, leptin, and ghrelin, and increased insulin resistance due to short sleep duration were found to increase the risks of obesity and diabetes [41–43]. In contrast, insomniacs were more likely to have insulin resistance than non-insomniacs. This study demonstrated that FSSG score was significantly correlated only with AIS score. Multivariate analysis showed that FSSG was an independent risk factor associated with insomnia, suggesting that GERD symptoms are responsible for insomnia in NAFLD patients, findings consistent with previously reported results [9, 17]. For example, sleep disorders, such as inability to sleep, difficult falling asleep, and awakening during the night, were observed in 56.3 % of patients with heartburn [9]. Similarly, we found that 61.3 % (19/31) of NAFLD patients with GERD had insomnia. A study in 134 Japanese patients with GERD found that FSSG score was significantly positively correlated with PSQI score [11]. GERD can affect sleep through two primary mechanisms. First, nighttime reflux, which occurs in 47–79 % of patients with GERD, can cause awakening during the night. Second, GERD can cause short, amnestic arousals (approximately 30 s), resulting in sleep fragmentation. However, recent studies also suggested that the link between GERD and sleep problems may be bidirectional. Sleep stage may influence the esophago-upper esophageal sphincter contractile reflex [44]. Sleep disturbance may reinforce the perception of intraesophageal acid [45]. The association of NAFLD with GERD has been assessed in only two studies, which reported that 37 and 51 % of patients with NAFLD had GERD symptoms [7, 8], percentages higher than observed in the present study. Plausible explanations of a lower prevalence (25 %) of GERD in our NAFLD patients were the difference of ethics, sex/age distribution, and the diagnostic method of GERD or NAFLD between previous studies [7, 8] and ours. Another explanation is the possibility that our NAFLD patients receiving dietary educations might avoid irregular diet habits, which are known to be the most significant risk factors for GERD symptoms [15]. In the future, sex- and age-matched controlled studies using a larger population is essential to draw conclusions. A recent study of Japanese patients with NAFLD found that GERD symptoms were significantly more severe in the group with higher than lower total cholesterol (T-CHO) and triglyceride (TG) levels [7]. In contrast, we observed no correlation between GERD symptoms and either T-CHO or TG. These conflicting results may be due to our inclusion of only patients with biopsy-proven NAFLD, who are not representative of the general population of patients diagnosed with NAFLD. In contrast, the patients included in the previous study were diagnosed with NAFLD by ultrasound [7]. Moreover, that study did not assess the association of insomnia with GERD symptoms. Thus, to our knowledge, our study is the first to clarify the relationship between insomnia and GERD symptoms in patients with NAFLD.

Previous studies suggested that acid suppression can improve sleep problems in GERD patients. For example, a prospective randomized clinical trial found that a significantly higher percentage of patients treated with esomeprazole than placebo showed resolution of GERD-related sleep disturbances [46]. RPZ treatment also significantly improved subjective indices of sleep quality over placebo [16]. Moreover, an 8-week course of RPZ treatment significantly decreased both FSSG and PSQI scores in Japanese patients [11]. Consistent with these findings, we found that treatment with RPZ significantly decreased both AIS and FSSG scores. These results also indicate that GERD symptoms are at least partly responsible for the occurrence of insomnia in NAFLD patients.

In addition to AIS score, GGT concentration was found to be an independent predictor of insomnia in patients with NAFLD. Serum GGT activity is a marker of oxidative stress [47]. The primary function of GGT is to maintain intracellular concentrations of glutathione, a critical antioxidant molecule. Thus, increased GGT activity can be regarded as a response to oxidative stress, aimed at increasing the intracellular concentration of glutathione. Although little is known about the relationship between insomnia and oxidative stress, a preliminary study showed that anti-oxidant activity was significantly lower and lipid peroxidation levels significantly higher in patients with primary insomnia than in controls [48]. Serum concentrations of GGT and 8-hydroxydeoxyguanosine are correlated in patients with NAFLD, indicating the occurrence of oxidative stress [49]. GGT has also been associated with the occurrence of metabolic syndrome, early atherosclerosis, and cardiovascular events [50]. Moreover, GGT concentrations are higher in patients with obstructive sleep

apnea syndrome (OSAS) than in controls [51] and have been associated with nocturnal arterial oxygen desaturation. Furthermore, continuous positive airway pressure treatment has been shown to decrease GGT, further suggesting that the increase in GGT is directly associated with OSAS. OSAS has also been associated with the presence and severity of NAFLD [52], suggesting that insomnia in patients with NAFLD is at least partly associated with the occurrence of OSAS. Our finding, that elevated GGT is an independent predictor of insomnia, suggests that these patients also have OSAS, although this was not directly evaluated in our patient population.

This study had several limitations. First, its design was cross-sectional, making it difficult to establish a cause–effect relationship, and suggesting the need for prospective studies. Second, the AIS is a self-administered questionnaire and subjective measure of insomnia, suggesting the need for assessment of objective sleep variables such as those obtained during polysomnography. Third, GERD was diagnosed based on FSSG scores alone, without endoscopic examination or 24 h pH monitoring. Because all participants were Japanese, there is a possibility that our results may not be applicable for NAFLD patients of other races or ethnic groups. The current study also did not assess the effects of smoking habits, mental status, dietary habit, and work environments. Additional studies, in larger populations, are needed to assess these variables.

In conclusion, we found that about 30 % of Japanese patients with biopsy-proven NAFLD have insomnia. GERD symptoms may be important in the development of insomnia. PPIs may be clinically useful for treating insomnia in NAFLD patients.

Conflict of interest Yuichiro Eguchi received a research grant from Bristol-Myers Squibb. Yoshito Itoh received commercial research funds from Dainippon Sumitomo Pharma Co., Ltd.

References

1. Angulo P. Nonalcoholic fatty liver disease. N Engl J Med. 2002;346:1221–31.
2. Ludwig J, Viggiano TR, McGill DB, Oh BJ. Non-alcoholic steatohepatitis. Mayo Clinic experiences with a hitherto unnamed disease. Mayo Clin Proc. 1980;55:434–8.
3. David K, Kowdley KV, Unalp A, Kanwal F, Brunt EM, Schwimmer JB. Quality of life in adults with nonalcoholic fatty liver disease: baseline data from the nonalcoholic steatohepatitis clinical research network. Hepatology. 2009;49:1904–12.
4. Kim CW, Yun KE, Jung HS, Chang Y, Choi ES, Kwon MJ, et al. Sleep duration and quality in relation to non-alcoholic fatty liver disease in middle-aged workers and their spouses. J Hepatol. 2013;59(2):351–7.
5. Hsieh SD, Muto T, Murase T, Tsuji H, Arase Y. Association of short sleep duration with obesity, diabetes, fatty liver and behavioral factors in Japanese men. Intern Med. 2011;50:2499–502.
6. Dew MA, Hoch CC, Buysse DJ, Monk TH, Begley AE, Houck PR, et al. Healthy older adults' sleep predicts all-cause mortality at 4 to 19 years of follow-up. Psychosom Med. 2003;65:63–73.
7. Fujikawa Y, Tominaga K, Fujii H, Machida H, Okazaki H, Yamagami H, et al. High prevalence of gastroesophageal reflux symptoms in patients with non-alcoholic fatty liver disease associated with serum levels of triglyceride and cholesterol but not simple visceral obesity. Digestion. 2012;86:228–37.
8. Miele L, Cammarota G, Vero V, Racco S, Cefalo C, Marrone G, et al. Non-alcoholic fatty liver disease is associated with high prevalence of gastro-oesophageal reflux symptoms. Dig Liver Dis. 2012;44:1032–6.
9. Kusano M, Kouzu T, Kawano T, Ohara S. Nationwide epidemiological study on gastroesophageal reflux disease and sleep disorders in the Japanese population. J Gastroenterol. 2008;43:833–41.
10. Jansson C, Nordenstedt H, Wallander MA, Johansson S, Johnsen R, Hveem K, Lagergren J. A population-based study showing an association between gastroesophageal reflux disease and sleep problems. Clin Gastroenterol Hepatol. 2009;7:960–5.
11. Fujiwara Y, Kohata Y, Kaji M, Nebiki H, Yamasaki T, Sasaki E, et al. Sleep dysfunction in Japanese patients with gastroesophageal reflux disease: prevalence, risk factors, and efficacy of rabeprazole. Digestion. 2010;81:135–41.
12. Johnson DA. Gastroesophageal reflux disease and sleep disorders: a wake-up call for physicians and their patients. Rev Gastroenterol Disord. 2005;5(Suppl 2):S3–11.
13. Mody R, Bolge SC, Kannan H, Fass R. Effects of gastroesophageal reflux disease on sleep and outcomes. Clin Gastroenterol Hepatol. 2009;7:953–9.
14. Orr WC. Review article: sleep-related gastro-oesophageal reflux as a distinct clinical entity. Aliment Pharmacol Ther. 2010;31:47–56.
15. Yamamichi N, Mochizuki S, Asada-Hirayama I, Mikami-Matsuda R, Shimamoto T, Konno-Shimizu M, et al. Lifestyle factors affecting gastroesophageal reflux disease symptoms: a cross-sectional study of healthy 19864 adults using FSSG scores. BMC Med. 2012;10:45.
16. Orr WC, Goodrich S, Robert J. The effect of acid suppression on sleep patterns and sleep-related gastro-oesophageal reflux. Aliment Pharmacol Ther. 2005;21:103–8.
17. Fujiwara Y, Kohata Y, Kaji M, Nebiki H, Yamasaki T, Sasaki E, et al. Sleep dysfunction in Japanese patients with gastroesophageal reflux disease: prevalence, risk factors, and efficacy of rabeprazole. Digestion. 2010;81:135–41.
18. Examination Committee of Criteria for 'Obesity Disease' in Japan; Japan Society for the Study of Obesity. New criteria for 'obesity disease' in Japan. Circ J. 2002;66:987–92.
19. Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. Report of the Committee on the Classification and Diagnostic Criteria of Diabetes Mellitus. J Jpn diabetes Soc. 2010;53:450–67.
20. Ogihara T, Kikuchi K, Matsuoka H, Fujita T, Higaki J, Horiuchi M, et al. The Japanese Society of Hypertension Guidelines for the Management of Hypertension (JSH 2009). Hypertens Res. 2009;32:3–107.
21. Kusano M, Shimoyama Y, Sugimoto S, Kawamura O, Maeda M, Minashi K, Kuribayashi S, Higuchi T, Zai H, Ino K, Horikoshi T, Sugiyama T, Toki M, Ohwada T, Mori M. Development and evaluation of FSSG: frequency scale for the symptoms of GERD. J Gastroenterol. 2004;39:888–91.
22. Yasaka S, Murakami K, Abe T, Anan J, Mizukami K, Tanahashi J, Okimoto T, Kodama M, Kudo Y, Kawasaki H, Fujioka T. Evaluation of esophageal function in patients with gastroesophageal reflux disease using transnasal endoscopy. J Gastroenterol Hepatol. 2009;24:1677–82.

23. Miyamoto M, Haruma K, Takeuchi K, Kuwabara M. Frequency scale for symptoms of gastroesophageal reflux disease predicts the need for addition of prokinetics to proton pump inhibitor therapy. J Gastroenterol Hepatol. 2008;23:746–51.
24. Furuta T, Shimatani T, Sugimoto M, Ishihara S, Fujiwara Y, Kusano M, et al. Investigation of pretreatment prediction of proton pump inhibitor (PPI)-resistant patients with gastroesophageal reflux disease and the dose escalation challenge of PPIs-TORNADO study: a multicenter prospective study by the Acid-Related Symptom Research Group in Japan. J Gastroenterol. 2011;46:1273–83.
25. Sakamoto Y, Inamori M, Iwasaki T, Lida H, Endo H, Hosono K, et al. Relationship between upper gastrointestinal symptoms and diet therapy: examination using frequency scale for the symptoms of gastroesophageal reflux disease. Hepatogastroenterology. 2010;57:1635–8.
26. Soldatos CR, Dikeos DG, Paparrigopoulos TJ. Athens Insomnia Scale: validation of an instrument based on ICD-10 criteria. J Psychosom Res. 2000;48:555–60.
27. Soldatos CR, Dikeos DG, Paparrigopoulos TJ. The diagnostic validity of the Athens Insomnia Scale. J Psychosom Res. 2003;55:263–7.
28. Matteoni CA, Younossi ZM, Gramlich T, Boparai N, Liu YC, McCullough AJ. Nonalcoholic fatty liver diseases: a spectrum of clinical and pathological severity. Gastroenterology. 1999;116:1413–9.
29. Kleiner DE, Brunt EM, Van Natta M, Behling C, Contos MJ, Cummings OW, Nonalcoholic Steatohepatitis Clinical Research Network, et al. Design and validation of a histological scoring system for nonalcoholic fatty liver disease. Hepatology. 2005;41:1313–21.
30. Brunt EM, Janney CG, Di Bisceglie AM, Neuschwander-Tetri BA, Bacon BR. Nonalcoholic steatohepatitis: a proposal for grading and staging the histological lesions. Am J Gastroenterol. 1999;94:2467–74.
31. Doi Y, Minowa M, Uchiyama M, Okawa M, Kim K, Shibui K, Kamei Y. Psychometric assessment of subjective sleep quality using the Japanese version of the Pittsburgh Sleep Quality Index (PSQI-J) in psychiatric disordered and control subjects. Psychiatry Res. 2000;97:165–72.
32. Kim K, Uchiyama M, Okawa M, Liu X, Ogihara R. An epidemiological study of insomnia among the Japanese general population. Sleep. 2000;23:41–7.
33. Yoshioka E, Saijo Y, Kita T, Satoh H, Kawaharada M, Kishi R. Effect of the interaction between employment level and psychosocial work environment on insomnia in male Japanese public service workers. Int J Behav Med. 2012. doi:10.1007/s12529-012-9230-9.
34. Yoshioka E, Saijo Y, Kita T, Satoh H, Kawaharada M, Fukui T, Kishi R. Gender differences in insomnia and the role of paid work and family responsibilities. Soc Psychiatry Psychiatr Epidemiol. 2012;47:651–62.
35. Monterrosa-Castro A, Marrugo-Flórez M, Romero-Pérez I, Chedraui P, Fernández-Alonso AM, Pérez-López FR. Prevalence of insomnia and related factors in a large mid-aged female Colombian sample. Maturitas. 2013;74:346–51.
36. Blümel JE, Cano A, Mezones-Holguín E, Barón G, Bencosme A, Benítez Z, et al. A multinational study of sleep disorders during female mid-life. Maturitas. 2012;72:359–66.
37. Yoshioka E, Saijo Y, Fukui T, Kawaharada M, Kishi R. Association between duration of daily visual display terminal work and insomnia among local government clerks in Japan. Am J Ind Med. 2008;51:148–56.
38. Utsugi M, Saijo Y, Yoshioka E, Horikawa N, Sato T, Gong Y, Kishi R. Relationships of occupational stress to insomnia and short sleep in Japanese workers. Sleep. 2005;28:728–35.
39. Nishitani N, Sakakibara H. Job stress factors, stress response, and social support in association with insomnia of Japanese male workers. Ind Health. 2010;48:178–84.
40. Kozaki T, Miura N, Takahashi M, Yasukouchi A. Effect of reduced illumination on insomnia in office workers. J Occup Health. 2012;54:331–5.
41. Spiegel K, Tasali E, Penev P, Van Cauter E. Brief communication: sleep curtailment in healthy young men is associated with decreased leptin levels, elevated ghrelin levels, and increased hunger and appetite. Ann Intern Med. 2004;141:846–50.
42. Spiegel K, Leproult R, L'hermite-Baleriaux M, Copinschi G, Penev PD, Van Cauter E. Leptin levels are dependent on sleep duration: relationships with sympathovagal balance, carbohydrate regulation, cortisol, and thyrotropin. J Clin Endocrinol Metab. 2004;89:5762–71.
43. Donga E, Van Dijk M, Van Dijk JG, Biermasz NR, Lammers GJ, van Kralingen KW, et al. A single night of partial sleep deprivation induces insulin resistance in multiple metabolic pathways. J Clin Endocrinol. 2010;95:2963–8.
44. Bajaj JS, Bajaj S, Dua KS, Jaradeh S, Rittmann T, Hofmann C, Shaker R. Influence of sleep stages on esophago-upper esophageal sphincter contractile reflex and secondary esophageal peristalsis. Gastroenterology. 2006;130:17–25.
45. Schey R, Dickman R, Parthasarathy S, Quan SF, Wendel C, Merchant J, et al. Sleep deprivation is hyperalgesic in patients with gastroesophageal reflux disease. Gastroenterology. 2007;133:1787–95.
46. Johnson DA, Orr WC, Crawley JA, Traxler B, McCullough J, Brown KA, Roth T. Effect of esomeprazole on nighttime heartburn and sleep quality in patients with GERD: a randomized, placebo-controlled trial. Am J Gastroenterol. 2005;100:1914–22.
47. Lee DH, Jacobs DR Jr. Serum gamma-glutamyltransferase: new insights about an old enzyme. J Epidemiol Community Health. 2009;63:884–6.
48. Gulec M, Ozkol H, Selvi Y, Tuluce Y, Aydin A, Besiroglu L, Ozdemir PG. Oxidative stress in patients with primary insomnia. Prog Neuropsychopharmacol Biol Psychiatry. 2012;37:247–51.
49. Irie M, Sohda T, Iwata K, Kunimoto H, Fukunaga A, Kuno S, et al. Levels of the oxidative stress marker γ-glutamyltranspeptidase at different stages of nonalcoholic fatty liver disease. J Int Med Res. 2012;40:924–33.
50. Kozakova M, Palombo C, Eng MP, Dekker J, Flyvbjerg A, Mitrakou A, et al. Fatty liver index, gamma-glutamyltransferase, and early carotid plaques. Hepatology. 2012;55:1406–15.
51. Barceló A, Barbé F, de la Peña M, Vila M, Pérez G, Piérola J, et al. Antioxidant status in patients with sleep apnoea and impact of continuous positive airway pressure treatment. Eur Respir J. 2006;27:756–60.
52. Musso G, Cassader M, Olivetti C, Rosina F, Carbone G, Gambino R. Association of obstructive sleep apnoea with the presence and severity of non-alcoholic fatty liver disease. A systematic review and meta-analysis. Obes Rev. 2013;14:417–31.

Semin Immunopathol (2014) 36:115–132
DOI 10.1007/s00281-013-0404-6

REVIEW

Microbiota and nonalcoholic steatohepatitis

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Received: 7 April 2013 / Accepted: 15 October 2013 / Published online: 14 December 2013

Abstract The recent rise in obesity-related diseases, such as nonalcoholic fatty liver disease and its strong association with microbiota, has elicited interest in the underlying mechanisms of these pathologies. Experimental models have highlighted several mechanisms connecting microbiota to the development of liver dysfunction in nonalcoholic steatohepatitis (NASH) such as increased energy harvesting from the diet, small intestine bacterial overgrowth, modulation of the intestinal barrier by glucagon-like peptide-2 secretions, activation of innate immunity through the lipopolysaccharide–CD14 axis caused by obesity-induced leptin, periodontitis, and sterile inflammation. The manipulation of microbiota through probiotics, prebiotics, antibiotics, and periodontitis treatment yields encouraging results for the treatment of obesity, diabetes, and NASH, but data in humans is scarce.

Keywords Nonalcoholic steatohepatitis (NASH) · Nonalcoholic fatty liver disease (NAFLD) · Microbiota · Inflammation · Insulin resistance

Abbreviations

ALF	Acute liver failure
ALT	Alanine aminotransferase
ASH	Alcoholic steatohepatitis
AST	Aspartate aminotransferase
ChREBP	Carbohydrate-responsive element-binding protein
DAMPs	Damage-associated molecular patterns
DM	Diabetes mellitus
Fiaf	Fasting-induced adipose factor
FFAs	Free fatty acids
GLP	Glucagon-like peptide
HCC	Hepatocellular carcinoma
HBV	Hepatitis B virus
HFD	High-fat diet
HMGB1	High-mobility group protein B1
HLA	Human leukocyte antigen
ICAM-1	Intercellular adhesion molecule-1
IL	Interleukin
LPL	Lipoprotein lipase inhibitor
IRS	Insulin receptor substrates
LPS	Lipopolysaccharide
MCD	Methionine choline-deficient
MyD88	Myeloid differentiation factor 88
NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
NF-κB	Nuclear factor kappa-B
PAMPs	Pathogen-associated molecular patterns
P. gingivalis	*Porphyromonas gingivalis*
RCT	Randomized controlled trial
SIBO	Small intestinal bacterial overgrowth
SI	Sterile inflammation
SREBP-1	Sterol-responsive element-binding protein
STAT3	Signal transducer and activator of transcription 3
TIR	Toll-IL-1 receptor
TLR	Toll-like receptor
TNF-α	Tumor necrosis factor-α
WT	Wild-type

This article is a contribution to the special issue on Metabolic Syndrome - Guest Editor: T. Miyazaki

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Introduction

Humans are colonized by residential microbes including bacteria, archaea, eukaryotes, and viruses [1]. Until recently, the properties of the microbiome of humans were largely unknown. The proportion of bacterial cells and genes present in the human body are estimated to be ~90 and >99 %, respectively. Initial colonization occurs at the time of birth, and humans progressively acquire $\sim 10^{14}$ bacterial cells at equilibrium, which remain for life. Recent studies of the human microbiome revealed that even healthy individuals differ remarkably in the microbes that occupy the gut, oral cavity, skin, and vagina. The Human Microbiome Project by the US National Institutes of Health has produced a 2.3-TB 16S ribosomal RNA metagenomic data set of over 35 billion reads taken from 690 samples from 300 US subjects, across 15 body sites [1]. Large-scale endeavors (for example, the Human Microbiome Project and the European project, MetaHIT) are already providing a preliminary understanding of the biology and medical significance of the human microbiome and its collective genes [3]. The aim of these projects is to characterize the compositional range of the "normal" microbiome of healthy individuals. Important questions concerning the commonalities and differences among healthy individuals in both microbial taxa and functional pathways are being addressed.

Nonalcoholic fatty liver disease (NAFLD) is an important cause of chronic liver injury in many countries [3–7]. Data collected from the USA showed that the prevalence of NAFLD has increased steadily in recent years, despite other diseases remaining at steady states [8]. NAFLD ranges from benign simple steatosis to steatohepatitis (including nonalcoholic steatohepatitis, NASH). This latter condition includes progressive fibrosis [9] and hepatocellular carcinoma [10–12]. The prevalence of obesity and its associated disorders, metabolic syndrome, have increased risk for many diseases such as NAFLD, atherosclerosis, and certain cancers. Moreover, studies on the relationship of intestinal microbial flora with obesity have identified profound changes in the composition and metabolic function of the intestinal microbiota in obese individuals [1, 13–15], which appear to enable the "obese microbiota" to extract more energy from the diet [16]. These facts suggest that microbiota could play an important role in obesity, NAFLD, and its related diseases. However, the relationship between this "obese microbiota" and the pathogenesis of NAFLD has not yet been elucidated. In this article, we will discuss current evidence regarding the potential role of microbiota in the development of NAFLD, with a special focus on inflammation and obesity-related metabolic disorders.

Inflammation and NASH

An interesting working model known as the "two-hit" theory postulates the progression from simple steatosis to NASH, fibrosis, or cirrhosis. The "first hit" consists of the accumulation of excessive hepatic fat owing to insulin resistance and inflow of free fatty acid (FFA) (Fig. 1). Often, this step is present in patients with metabolic syndrome, and although it is not sufficient to cause NASH, it can predispose the liver to chronic inflammation. Oxidative stress caused by reactive oxygen species (ROS), gut-derived lipopolysaccharide (LPS), and soluble mediators synthesized from immune system cells and adipose tissue cells have been

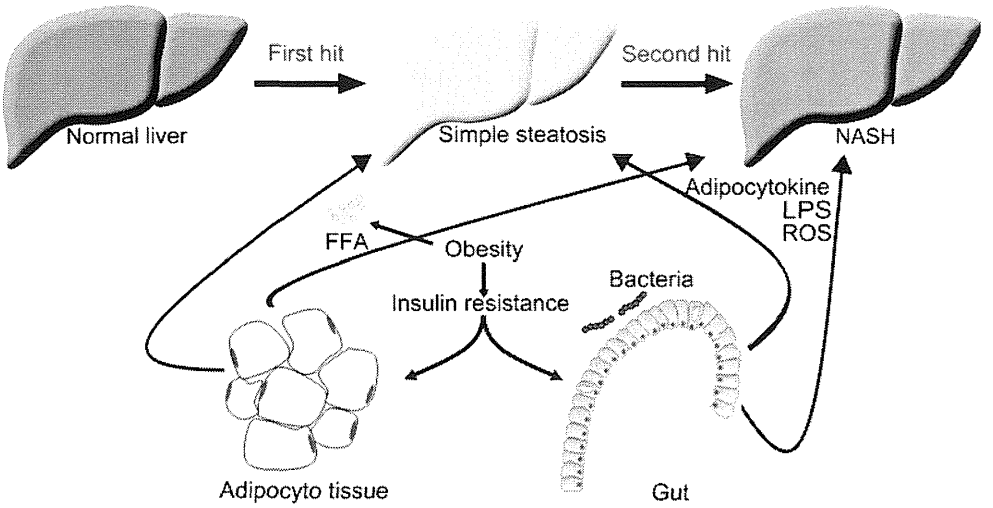

Fig. 1 Multifactorial complex interactions leading to hepatic steatosis and its progression. A liver loaded with lipids consisting primarily of triglycerides (*first hit*) might reflect a benign process because they might exert mostly protective effects. When the capacity of peripheral and central organs to detoxify aggressive lipids, including free fatty acid (*FFA*) (*second hit*) fails, lipotoxic attack of the liver might begin. Moreover, adipocytokine, lipopolysaccharide (*LPS*), and reactive oxygen species (*ROS*) may enhance the first and/or second hits, resulting in progression of steatohepatitis pathogenesis

indicated as risk factors for the "second hit" [17, 18] (Fig. 1). Although the model of the "two-hit theory" quickly spread through the scientific world, it seems obvious that different factors are necessarily interacting. In the last few years, studies in animal models of NAFLD have provided new insights into the molecular and physiologic alterations responsible for the switch from steatosis to steatohepatitis. In this regard, several groups demonstrated that in NAFLD progression, complex multifactorial interactions between genetic determinants, nutritional factors, and dysmetabolism participate in the development of hepatocellular damage and progressive liver disease. Lipotoxic effects of FFAs and lipid intermediates impair the normal functions of liver cell organelles involved in the production of ROS, the activation of pro-inflammatory defense programs, and ultimately apoptosis, by mechanisms that are not fully understood. Toxic lipids and cytokine release contribute to impaired insulin signaling, which in turn causes diminished very-low-density lipoprotein (VLDL) assembly and liver secretion, involving insufficient regulation of important transcription factors required for lipogenesis [19].

Inflammation is a physiological response of an organism to harmful physical, chemical, or biological stimuli. The response usually concludes with the reestablishment of homeostasis. It involves the coordinated action of many cell types and mediators, whose intervention depends on the nature of the initial stimulus and ensuing responses. The normal acute inflammatory response involves the delivery of plasma components and leukocytes to the site of insult and is initiated by tissue-resident macrophages and mast cells leading to a production of different types of inflammatory mediators (cytokines, chemokines, vasoactive amines, eicosanoids, and products of proteolytic cascades) [20]. The inflammatory state that accompanies the metabolic syndrome shows a quite peculiar presentation, as it is not accompanied by infection or sign of autoimmunity, and no massive tissue injury seems to occur. Furthermore, the dimensions of inflammatory activation are not large, and so it is often called "low-grade" chronic inflammation. Other researchers have attempted to name this inflammatory state "metainflammation," meaning metabolically triggered inflammation [21] or "parainflammation" as a term to define an intermediate state between basal and inflammatory states [22]. Whatever the term used, the inflammatory process that characterizes the metabolic syndrome has unique features, and its mechanisms are far from being fully understood [24]. Metabolic syndrome, particularly NASH, is thought to develop through "metainflammation." Indeed, chronic low-grade inflammation is an important contributing factor in NASH pathogenesis [24, 25].

The liver is continually exposed to gut-derived factors including bacteria and bacterial components because the portal vein is the direct venous outflow of the intestine. The liver is an important site for bacterial phagocytosis and clearance as it contains the largest population of tissue macrophages. Activated Kupffer cells, resident macrophages of the liver, exposed to pro-inflammatory mediators such as LPS, membrane components of gram-negative bacteria, or other bacterial products, are the major source of inflammatory mediators including pro-inflammatory cytokines, chemokines, and reactive oxygen/nitrogen species, which contribute to liver injury [26]. Through pattern recognition receptors, including Toll-like receptors (TLRs), the innate immune system recognizes conserved pathogen-associated molecular patterns (PAMPs) [27]. The healthy liver expresses low mRNA levels of TLRs such as TLR1, TLR2, TLR4, TLR6, TLR7, TLR8, TLR9, and TLR10, implying a high tolerance of the liver to TLR ligands from the microbiota, to which it is constantly exposed. Signaling through TLRs plays a major role in the physiology and pathophysiology of the liver [28]. LPS is a potent activator of innate immune responses through binding to the TLR4 complex. TLR4 is expressed by Kupffer cells, hepatic stellate cells, hepatocytes, biliary epithelial cells, sinusoidal endothelial cells, and hepatic dendritic cells, which are consequently responsive to LPS [28]. There is a positive correlation between liver dysfunction and the occurrence of bacterial translocation and increased LPS. Furthermore, the clearance of LPS from the circulation is decreased in states of hepatic dysfunction, such as cirrhosis [29]. Downstream targets of TLR4 signaling are determined by selective recruitment of cytosolic sorting and signaling adaptor proteins via interactions between Toll-IL-1 receptor (TIR) domains [30–32]. Thus, TLR4 activation may engage myeloid differentiation factor 88 (MyD88) and TIR domain-containing adaptor protein or MyD88 adaptor-like factors, leading to the activation of nuclear factor kappa-B (NF-κB) and AP-1 transcription factors [33–35]. There is substantial evidence that TLR4-mediated cellular events escalate liver injury in steatosis [33, 35]. Recent studies indicated that TLR4 sorting specificity might reflect the etiology of fatty liver disease. Due to the ubiquitous presence of TLR4 among various types of liver cells, the specific role of Kupffer cells in differential activation of TLR4 pathways remains to be determined. It must also be noted that endogenous ligands such as certain FFA and other alarmins may also be linked to TLR4 sorting specificity, a question particularly relevant to NAFLD.

Kupffer cells produce tumor necrosis factor-α (TNF-α) and interleukin (IL)-10 in response to physiological concentrations of LPS [36–38]. TNF-α is a proinflammatory cytokine that activates various signal transduction cascades, including many of the pathways that are discussed below, as critical inhibitors of insulin action. It is overexpressed in the adipose tissues and livers of obese rodents and humans, and its concentration is reduced after weight loss. Geoffrey et al. showed that TNF-α and NF-κB activation was essential for hepatic inflammatory recruitment in steatohepatitis by using methionine choline-deficient (MCD) diet-fed TNF-α and

TNF-receptor 1 knockout animals, and in vivo transfection of wild-type (WT) mice with nondegradable mutant-IκB [39]. Furthermore, such NF-κB activation occurred independently of TNF-α. Other studies using the MCD dietary model produced conflicting findings; curcumin, which blocks oxidative stress-mediated NF-κB activation, provided protection [40], but TNF-α antiserum reduced liver injury in rats administered the MCD diet [41]. Tomita et al. demonstrated that TNF-receptor knockout mice were protected against liver fibrosis in their MCD experiments [42].

Alternatively, activated macrophages (also termed M2 as opposed to the classical M1 or proinflammatory phenotype), including Kupffer cells, represent another critical pathway for the resolution of inflammatory responses [43]. The coordinated program of alternative activation is primarily stimulated by Th2 cytokines IL-4 and IL-13 and characterized by the cell surface expression of M2 signature genes such as the mannose receptor, arginase-1, and dectin-1 [43]. There is evidence that steatosis promotes Th1 polarization of the cytokine balance, which favors the innate or classic activation of macrophages in NAFLD. Thus, in experimental and human NAFLD alike, the pool of hepatic natural killer T cells (NKT) is reduced, and liver tissue levels of Th1 cytokines, such as TNF-α, IL-12, IL-18, and interferon-γ, are elevated [44–47]. These studies suggest that microbiota-induced "metainflammation" associated with pro-inflammatory cytokines is critical in the pathogenesis of NASH, but a complex and still poorly characterized interaction between microbiota and the innate immune system for "metainflammation" may be involved in metabolic dysfunction.

Intestinal microbiota and NASH

The human gut contains an immense number of microorganisms, collectively known as the intestinal microbiota. This community consists of at least 10^{13} citizens, is dominated by anaerobic bacteria, and includes 500–1,000 species whose collective genomes are estimated to contain 100 times more genes than our own human genome [47, 48]. The duodenum and proximal jejunum normally contain small numbers of bacteria, usually lactobacilli and enterococci, gram-positive aerobes or facultative anaerobes ($<10^4$ organisms/mL). Coliforms may be transiently present ($<10^3$ bacteria/mL), and anaerobic *Bacteroides* are not found in the jejunum in healthy people. Up to one third of jejunal aspirates might be sterile in healthy volunteers. The distal ileum is a transition zone between sparse populations of aerobic bacteria of the proximal small intestine and very dense populations of anaerobic microorganisms in the large bowel [49–51]. The epithelial surface of the small intestine in a healthy human is not colonized. Occasional groups of bacteria can be found in low concentrations within the lumen. Bacteria do not form clusters and spatial structures, and the luminal contents are separated from the mucosa by a mucus layer [52].

Important studies on the relationship of intestinal microbial flora with obesity have identified profound changes in the composition and metabolic function of the intestinal microbiota in obese individuals (obese microbiota), as described above. Moreover, these studies demonstrated that intestinal microbiota interact with host epithelial cells to indirectly control energy expenditure and storage [13], and activated inflammatory responses in NASH pathogenesis. Several reports demonstrating an association between microbiota and NASH are shown in Table 1.

Intestinal microbiota and metabolic disorders

Intestinal microbiota benefits the host in numerous ways, among them the capability to extract calories from otherwise indigestible common polysaccharides in the diet via enzymes such as glycoside hydrolases and others that are not encoded within the human genome (Fig. 2) [53–55]. The first evidence that intestinal microbiota may be associated with body weight and composition was reported by Bachhed et al. [13]. They analyzed germ-free mice and conventionally raised mice that were allowed to acquire intestinal microbiota from birth to adulthood [13]. Compared to germ-free mice, conventional mice developed more adipose tissue, a higher percentage of body fat, and a twofold increase in hepatic triglyceride content, accompanied by increased hepatic mRNA expression of sterol-responsive element-binding protein (SREBP-1) and carbohydrate-responsive element-binding protein (ChREBP), two nuclear positive regulators of lipogenic enzymes [13], despite eating smaller amounts of the same diet (Fig. 2). Mice hosting intestinal microbiota also had higher levels of leptin and insulin resistance [58]. When they transplanted normal microbiota harvested from the distal intestine of conventionally raised mice into adult germ-free mice, a 57 % increase in body fat content and insulin resistance was observed [56] associated with decreased intestinal expression of fasting-induced adipose factor (Fiaf), also known as angiopoietin-like factor, a circulating lipoprotein lipase (LPL) inhibitor. This favored fatty acid uptake and adipose tissue expansion (Fig. 2) [56]. Conventionalization of germ-free mice suppressed the expression of Fiaf in gut epithelial cells [13]. Increased adipocyte LPL activity resulted in increased cellular uptake of fatty acids and adipocyte triglyceride accumulation (Fig. 2). Germ-free *Fiaf*−/− mice have the same total body fat weight as conventionalized mice, suggesting Fiaf is a mediator of microbial regulation of energy storage [13]. In contrast, mice fed a high-fat diet (HFD) complemented with *Lactobacillus paracasei* exhibited significantly reduced body fat, paralleled by increased circulating levels of Fiaf [57]. *L. paracasei* upregulated Fiaf

Table 1 Animal and clinical studies regarding microbiota and NAFLD

Animal studies			
Phenomenon	Model	Result	Reference
Small intestinal bacterial overgrowth (SIBO)	Sprague Dawley rat fed HFD	HFD induced alterations of intestinal microbiota, and antibiotics decreased liver dysfunction and serum TNF-α levels	[98]
Increase in intestinal permeability	Leptin-deficient (*ob/ob*) and hyperleptinemic (*db/db*) mice	*Ob/ob* and *db/db* mice showed lower intestinal resistance and higher circulating levels of inflammatory cytokines and portal endotoxemia, compared with lean control mice	[107]
	C57bl6/J mice fed HFD and *ob/ob* mice	HFD strongly increased intestinal permeability and reduced the expression of genes coding for proteins of the tight junctions	[109]
Hyperresponsivity to endotoxin	*C57bl6/J* fed HFD, *ob/ob* and *db/db* mice	HFD-induced steatosis mice showed hepatic hyperresponsivity to low-dose endotoxin, compared with *ob/ob*, *db/db*, and lean control mice	[120]
	C57bl/6J mice fed HCD	HCD increased the sensitivity of mice to endotoxin without affecting its plasma level	[121]
Periodontitis	*C57bl6/J* mice fed HFD plus *P. gingivalis*	Infection of *P. gingivalis* on NAFLD model mice accelerated the NAFLD progression	[155]
Clinical studies			
Phenomenon	Design	Result	Reference
Small intestinal bacterial overgrowth (SIBO)	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Two groups of patients: (1) $n=23$ patients with NASH, (2) $n=23$ control subjects	SIBO and serum TNF-α levels were increased in patients with NASH, compared with control subjects	[97]
	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Two groups of patients: (1) $n=12$ patients with NASH, (2) $n=11$ control subjects	SIBO was not different between patients with NASH and control subjects	[95]
	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Three groups of patients: (1) $n=35$ patients with NASH, (2) $n=27$ patients with celiac disease, (3) $n=24$ control subjects	Patients with NAFLD had a higher prevalence of SIBO, compared with control subjects	[108]
Increase in intestinal permeability	See above	Intestinal permeability was not different between patients with NASH and control subjects	[97]
	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Three groups of patients: (1) $n=10$ patients with NASH, (2) $n=6$ patients with simple steatosis, (3) $n=12$ control subjects	Intestinal permeability was increased in patients with NASH, compared with control subjects and patients with simple steatosis	[108]
	See above	Patients with NAFLD had significantly increased intestinal permeability, compared with control subjects	[108]
Hyperresponsivity to endotoxin	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Three groups of patients: (1) $n=57$ patients with NASH, (2) $n=35$ patients with simple steatosis, (3) $n=25$ control subjects	Hepatic CD14 expression, which is an important inflammatory response regulatory factor and enhancer of LPS, was increased in patients with NASH, compared with patients with simple steatosis and control subjects	[120]
Periodontitis	Cross-sectional, single-center, nonrandomized, noncontrolled pilot study. Three groups of patients: (1) $n=102$ patients with NASH, (2) $n=48$ patients with simple steatosis, (3) $n=60$ control subjects	The detection frequency of periodontitis in patients with NASH was markedly higher than in non-NAFLD subjects	[155]

HCD high-cholesterol diet, *HFD* high-fat diet, *NASH* nonalcoholic steatohepatitis, *NAFLD* nonalcoholic fatty liver disease, *SIBO* small intestinal bacterial overgrowth, *TNF* tumor necrosis factor

expression in colonic epithelial cell lines, and oral inoculation of germ-free mice with this species resulted in increased circulating Fiaf levels [57]. Fiaf also induces peroxisomal proliferator activated receptor gamma coactivator 1α (PGC1-α) that regulates the expression of enzymes involved in fatty acid oxidation (Fig. 2) [58]. Therefore, Fiaf appears to have an important role in central regulation of energy metabolism, leading to exacerbation of insulin resistance.

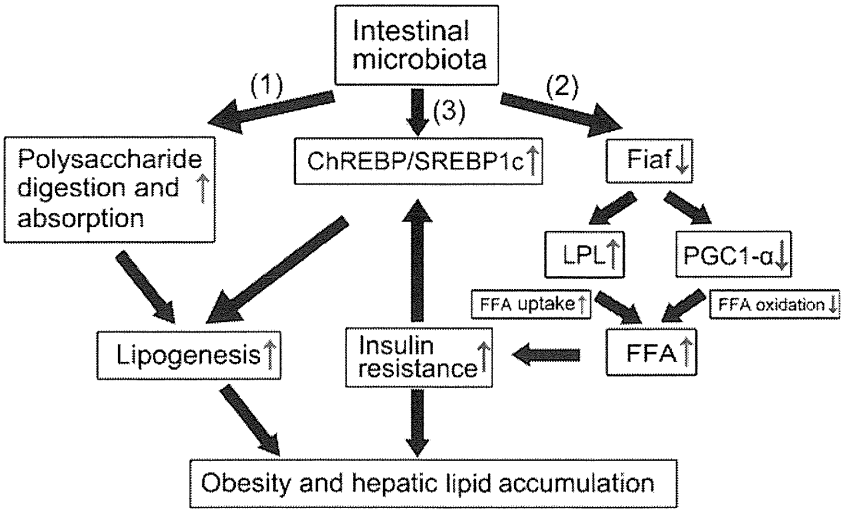

Fig. 2 Proposed mechanisms of the effects of intestinal microbiota on host metabolic disorder. (*1*) The digestion of polysaccharides by microbial enzymes leads to increased hepatic de novo lipogenesis. (*2*) The decreased expression of fasting-induced adipose factor (*Fiaf*) leads to increased lipoprotein lipase (*LPL*) activity, resulting in increased hepatic uptake of free fatty (*FFA*) acid and decreased expression of peroxisomal proliferator-activated receptor coactivator-1a (*PGC-1α*). These cause decreased hepatic oxidation of FFA. (*3*) The hepatic expression of sterol-responsive element-binding protein (*SREBP-1*) and carbohydrate-responsive element-binding protein (*ChREBP*) is increased through insulin resistance, following increased de novo lipogenesis. These effects lead to obesity and hepatic lipid accumulation

Insulin resistance and hyperinsulinemia were the most closely associated laboratory findings with the presence of NAFLD in a large series of patients [59–61]. NAFLD prevalence is increased in individuals with impaired glucose tolerance (43 %) and those with newly diagnosed diabetes mellitus (DM) (62 %) [62]. In a prospective study of 100 patients with type 2 DM, the incidence of hepatic steatosis was 49 %, confirming this strong independent risk factor for NAFLD [63]. In Japan, the prevalence of metabolic syndrome and DM among patients with NAFLD was estimated at 25.4 and 16.2 %, respectively [64]. The association of both conditions is related with more aggressive disease and increasing mortality [65, 66]. Targher et al. described increased prevalence of NAFLD and its association with cardiovascular disease in diabetic patients, independent of other confounding factors [67]. The occurrence of insulin resistance in the liver, characterized by reduced insulin-suppressing effects in hepatic glucose production, aggravated peripheral insulin resistance and contributed to hepatic lipogenesis [68]. Consequently, hyperinsulinemia caused increased hepatic synthesis of FFAs and decreased synthesis of apolipoprotein B-100, leading to triglyceride accumulation in the liver [69]. Thus, elevated FFA levels caused by insulin resistance in adipocytes lead to the decreased suppression of lipolysis by insulin [70]. The liver is not only an end organ for the effects of insulin resistance, but also regulates carbohydrate homeostasis, under the control of insulin signaling through its specific receptor [71]. The insulin receptor belongs to a subfamily of tyrosine kinase receptors including insulin-like growth factor-1 and insulin-related receptor [72]. When insulin binds its receptor, insulin receptor substrate (IRS)-1 and IRS-2 are phosphorylated and mediate insulin signaling in the liver, leading to the recruitment and activation of IRS by tyrosine phosphorylation, which recruits signaling molecules containing Src homology-2 domains, including phosphatidylinositol 3-kinase, growth factor receptor-bound protein 2, and SH2 domain containing tyrosine phosphatase. In turn, this activates downstream effectors that mediate the metabolic effects of insulin [73, 74]. In addition, IRS proteins undergo serine phosphorylation, which attenuates insulin signaling by decreasing insulin-stimulated tyrosine phosphorylation [75, 76]. Tyrosine phosphatases and serine kinases including c-Jun N-terminal kinase [71, 77], protein kinase C [78], inhibitor κB kinase complex [76], and mitogen-activated protein kinase are involved in these pathways [79]. Depending on which pathway is impaired, elevated circulating levels of insulin and glucose may play a crucial role in pathogenesis of NASH by hepatocyte injury.

Small intestinal bacterial overgrowth

Qualitative or quantitative imbalances of complex intestinal microbiota might have serious health consequences for a macroorganism, including small intestinal bacterial overgrowth syndrome (SIBO). Initial evidence for an altered microflora associated with obesity came from studies in the leptin-deficient *ob/ob* mouse model. 16S rRNA sequencing of the distal intestinal microbiota of *ob/ob* mice, lean *ob/+*, and wild-type siblings and their *ob/+* mothers, all fed the same diet, revealed that *ob/ob* mice exhibit a major reduction in the abundance of *Bacteroidetes* and a proportional increase in

Firmicutes [14]. Feeding of a high-fat/high-polysaccharide diet to genetically WT rodents led to similar microbial changes and SIBO [80]. Confounding factors affecting microbial composition and function may include diet; the use of antibiotics, which substantially reduces bacterial diversity [81]; and effects related to the genetic background of animal models [82]. Consistent with animal models, Ley, et al. observed analogous differences with an increase in the ratio of *Firmicutes/Bacteroidetes* in the distal intestinal microbiota in human obesity [83]. Another study demonstrated that *Firmicutes* were dominant in lean and obese individuals and decreased in three patients undergoing Roux-en-Y gastric bypass surgery [84]. In contrast to earlier studies, Zhang et al. [85] described that *Prevotellaceae*, a subgroup of *Bacteroidetes*, were significantly enriched in obese individuals, again raising the potentially important issue of diet as a confounding factor. Patients in the Ley study [83] were either on a fat-restricted or carbohydrate-restricted diet, whereas in the Zhang study, researchers did not limit dietary components. Another study also described a decrease of *Bacteroidetes* in obesity and an increase in *Firmicutes* [86]. Overweight pregnant patients also have reduced numbers of *Bifidobacteria* and *Bacteroidetes*, but increased numbers of certain *Firmicutes* or *Proteobacteria* [87].

The overall prevalence of SIBO in the public is unknown. In general, SIBO is substantially underdiagnosed. Some patients may not seek health care, or SIBO may not be properly diagnosed by medical investigations. SIBO might be asymptomatic or with nonspecific symptoms only, or all symptoms might be incorrectly ascribed to the underlying disease (leading to SIBO). Of course, diagnostic yield also depends on the methods used for investigation. According to different studies investigating small sets of clinically healthy people as controls, findings consistent with SIBO were found in 2.5 to 22 % [88–96]. Thus, SIBO may coexist with NASH (Table 1). Wan et al. reported that excessive multiplication of *Escherichia coli* coexisted in NASH rats, consistent with previous studies. This suggested SIBO may be one of many factors important in the pathogenesis of NASH, as antibacterial treatment could alleviate the severity of NASH (Table 1) [97]. In addition, levels of ALT increased or decreased relative to serum levels of TNF-α [97]. This strongly supported TNF-α as an important mediator for the promotion of NASH by SIBO. Generally, endotoxemia is thought to be a link between SIBO and elevated TNF-α levels [98, 99]. Furthermore, Wigg et al. found a higher prevalence of SIBO (11/22, 50 %) in patients with NASH, compared to healthy control subjects (5/23, 22 %) (Table 1) [97]. Higher values for the xylose–lactulose test in patients with NASH correlated with higher serum levels of TNF-α. However, they were not associated with increased serum endotoxin [97]. In another study of NASH, SIBO was diagnosed in half of the patients (6/12) but only in one subject (1/11, 9 %) in the healthy control group [100]. Treatment with ciprofloxacin suppressed bacterial overgrowth, increased serum insulin, and decreased endogenous ethanol production but did not influence serum acetylated ghrelin levels (half values compared to controls). Changes in fasting insulin and ethanol following ciprofloxacin suggested these parameters might be influenced by small intestinal bacterial activity. In an experimental rat model of NASH, there was a slower transit time and higher quantity of coliform bacteria (*E. coli*) present. Treatment with gentamicin (cidomycin) accelerated the transit time, decreased TNF-α levels, and alleviated severity of liver involvement in experimental animals [101]. Thus, SIBO might play an important role in the pathogenesis of NASH (Fig. 3). However, despite these findings, the impact of increased SIBO during NASH progression remains controversial. Sabate et al. reported that SIBO was not associated with the frequency of NASH in clinical study [95]. Thus, additional studies are required to clarify the involvement of SIBO in NASH pathogenesis.

Intestinal permeability and endotoxin

The idea that increased intestinal permeability and intestinal flora might contribute to the development of several diseases was first suggested in 1890 [102]. The occurrence of cross-talk between the gut and the liver is an intriguing hypothesis that could explain the hepatobiliary changes associated with several inflammatory and infectious intestinal diseases such as inflammatory bowel disease, celiac disease, and infections caused by *Salmonella* and *Yersinia* amongst others [103]. Evidence supporting a role for the liver–gut axis in the pathogenesis of NASH has been slowly accumulating [104–106].

Obesity may increase intestinal permeability via disorders of intestinal barrier integrity [106–108]. A HFD may increase intestinal translocation of endotoxin in mice (the so-called metabolic endotoxemia) and reduce enteric *Bifidobacteria* [109, 110], a group of bacteria that may lower intestinal LPS levels to improve mucosal barrier function [107]. Mechanisms regulating intestinal barrier integrity may also modulate the extent of endotoxinemia [106–108, 111]. Glucagon-like peptide (GLP)-2, a 33-amino-acid peptide with intestinotrophic functions, cosecreted with GLP-1 by enteroendocrine L cells, may be a key modulator of intestinal barrier function. Recently, Cani et al. assessed the effect of a prebiotic fermentable oligofructose on intestinal microbiota composition, intestinal permeability, and hepatic and systemic inflammation in obese *ob/ob* mice (Table 1) [112]. The prebiotic-supplemented diet increased the intestinal proportions of *Lactobacilli* and *Bifidobacteria*, increased the expression of epithelial tight-junction proteins occludin and zonulin-1 (restoring normal intestinal permeability), and reduced systemic endotoxinemia as well as hepatic inflammation and oxidative stress. These effects were

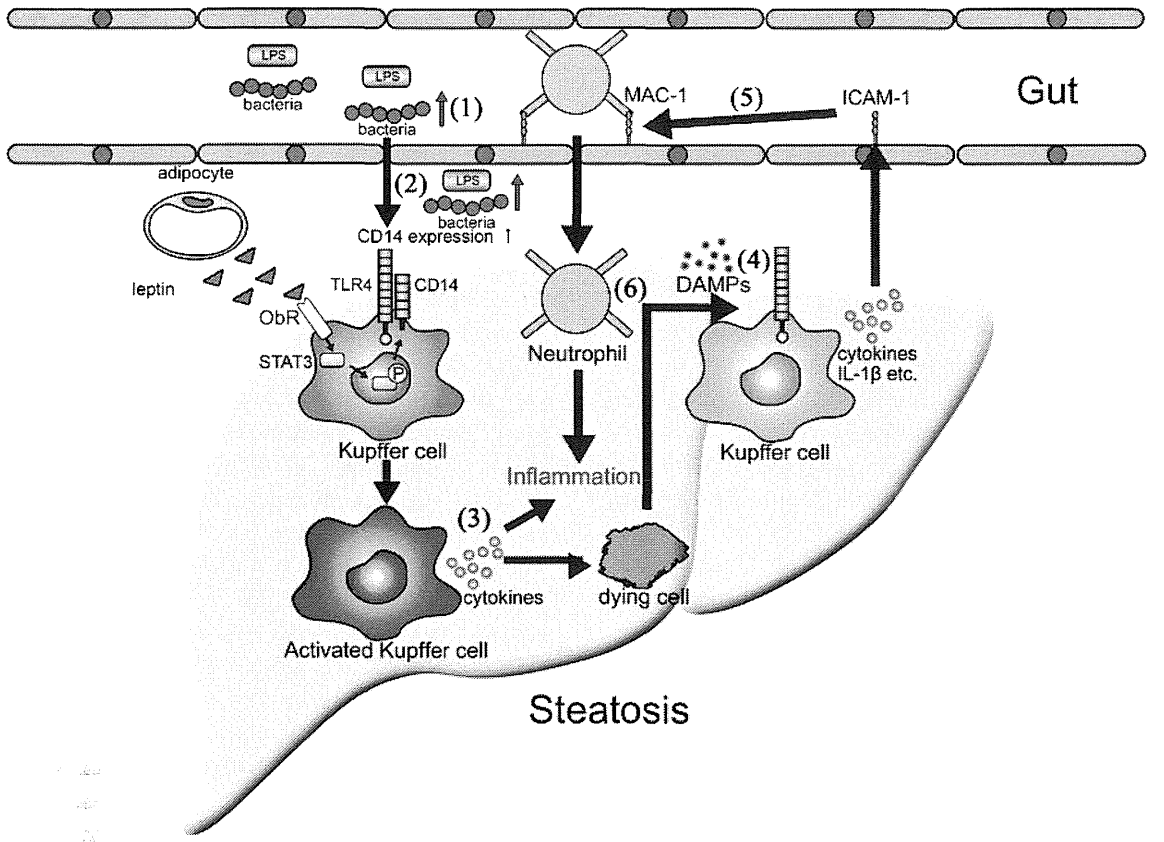

Fig. 3 Proposed mechanisms of intestinal microbiota-induced inflammation in the liver. (*1*) Small intestinal bacterial overgrowth and (*2*) increased intestinal permeability induce the translocation of bacteria and endotoxin. (*3*) Obesity-mediated increased leptin levels induce hyperreactivity against low-level bacteria and endotoxin, causing increased inflammation in steatosis. (*4*) Tissue damage and dying cells result in the release of damage-associated molecular patterns (*DAMPs*) that stimulate local intravascular sentinel cells, including Kupffer cells, to produce interleukin (IL)-1β, which induces upregulation of intercellular adhesion molecule-1 (*ICAM-1*) on sinusoidal endothelial cells (*5*). Neutrophils are then recruited via β2 integrin (*Mac-1*)-dependent adhesion to endothelial ICAM-1. (*6*) Finally, neutrophils are recruited to the liver

associated with increased intestinal GLP-2 levels, were mimicked by the administration of a GLP-2 agonist, and were prevented by pretreatment with a GLP-2 antagonist. These findings suggested GLP-2 might link intestinal microbiota, intestinal permeability, and systemic endotoxinemia and inflammation.

An alternative pathway for LPS absorption from the gut may involve chylomicron secretion from enterocytes, rather than loss of intercellular tight-junction integrity. Studies using cell cultures or animal models suggested that endotoxin is actively secreted into the blood with chylomicrons, and that inhibition of chylomicron synthesis blocks blocked endotoxin secretion [113]. Collectively, these data strongly connect gut-derived endotoxin, via disorder of intestinal barrier integrity and increase in chylomicron secretion from enterocytes, to the pathogenesis of NASH (Fig. 3).

Hyperresponsivity to endotoxin

Previous studies showed that gut-derived bacterial endotoxin may play a role in the progression of disease from simple steatosis to steatohepatitis in NASH [97, 98, 100, 106–108, 114–116]. Despite these findings, the impact of increased endotoxinemia during NASH progression remains controversial. Namely, it is unclear whether serum endotoxin levels are significantly higher in NASH patients than in control subjects or patients presenting with simple steatosis. Harte et al. reported that serum endotoxin levels were elevated in NAFLD patients compared with control subjects. However, Loguercio et al. showed endotoxemia was absent in all NAFLD patients tested [115, 117]. At present, there is general agreement that mild portal endotoxemia can be detected in healthy subjects due to gut-derived bacterial endotoxin [118]. However, the levels of portal endotoxemia observed under healthy conditions do not usually cause liver dysfunction [119]. We therefore propose it may be necessary to consider a low-level endotoxin-mediated mechanism for the progression of NASH. Here, we hypothesize that responsiveness against gut-derived bacterial endotoxin might be enhanced under simple steatosis compared with that of the healthy liver. Indeed, our own data show that responsivity against low-dose LPS was enhanced in HFD-induced murine

steatosis and that low-dose LPS led to liver injury and severe hepatic fibrosis in HFD-fed mice, but not in chow-fed mice (Table 1) [120]. Previous studies also showed that a high cholesterol diet increased the sensitivity of mice to LPS without affecting plasma levels, supporting our suggestion (Table 1) [121]. Next, we investigated the mechanisms for enhancing responsivity against low-dose LPS in HFD-induced murine steatosis compared with chow-fed murine healthy livers, and showed that CD14 is overexpressed in the liver of HFD-fed WT mice compared to chow-fed WT mice [120]. Our data clearly indicate that CD14-positive cells are specifically identified as Kupffer cells and that increased CD14-positive Kupffer cell numbers contributed to enhanced responsivity against low-dose LPS in simple steatosis. This suggests that hyperresponsivity against low-dose bacterial endotoxin is regulated by the expression of CD14 in Kupffer cells during simple steatosis [120]. CD14 is an important regulatory factor in LPS-induced inflammation and enhances the LPS effects in Kupffer cells [122–127]. In addition, a previous report showed that promoter polymorphisms of CD14 are a risk factor for human NASH [128]. Therefore, increased expression of CD14 is closely related to the pathogenesis of NASH. Indeed, our data showed that CD14 mRNA expression levels were much higher in NAFLD patients, including NAFL and NASH, than those in control subjects [120]. Therefore, hepatic CD14 may be an important factor in the development of NASH by enhancing hepatic inflammation against gut-derived bacterial endotoxin. We also investigated leptin-dependent increases in hepatic CD14 expression using leptin-deficient *ob/ob* mice and leptin receptor-deficient *db/db* mice. We found that both leptin-deficiency and leptin receptor deficiency led to decreased hepatic CD14 expression, resulting in decreased responsivity to LPS, despite mice exhibiting severe obesity and steatosis. In contrast, HFD-fed WT mice exhibited severe obesity and steatosis with increased hepatic CD14 expression via increased serum leptin levels and activation of leptin receptor and signal transducer and activator of transcription 3 (STAT3) signaling in the liver, causing enhanced LPS responsivity. Furthermore, exogenously administered leptin in chow-fed WT mice caused overexpression of hepatic CD14 despite a lack of obesity and steatosis, resulting in enhancing LPS responsivity. Increased CD14 expression was also observed in RAW264.7 cells, a murine monocyte/macrophage cell line, and Kupffer cells isolated from chow fed WT mice treated with leptin in vitro. Furthermore, the increase in CD14 expression in RAW246.7 cells was decreased after STAT3 inhibitor administration. Thus, leptin and STAT3 signaling may increase responsivity to gut-derived, low-dose bacterial endotoxin even in the healthy liver via an increase in CD14-positive Kupffer cells, irrespective of steatosis.

In humans, increased serum leptin levels are generally associated with obesity, visceral fat accumulation, and steatosis [129, 130], and patients with NAFLD are generally often obese and with hyperleptinemia. We also observed that serum leptin and hepatic CD14 expression levels in NAFLD patients were much higher than in control subjects. Similarly, the levels in NASH patients were much higher than in patients with simple steatosis. Significant positive correlations between serum leptin levels and hepatic CD14 expression levels were observed [120]. These results clearly indicate that serum leptin levels and hepatic CD14 expression are closely associated with the onset of NAFLD and its progression to NASH. In general, simple steatosis is considered to have progressed to NASH when factors such as oxidative stress, proinflammatory cytokines, and gut-derived high-level endotoxin are present in addition to conditions that enhance responsivity to endotoxin via increased hepatic CD14 expression. Under these circumstances, hepatic TNF-α expression may play an important role in liver inflammation and fibrosis. We observed that hepatic TNF-α expression was greater in NASH than simple steatosis, but not between simple steatosis and control subjects [120]. Thus, leptin-induced hepatic CD14 expression may enhance hepatic responsivity to gut-derived bacterial endotoxins, even at low levels, resulting in the progression from simple steatosis to NASH (Fig. 3).

Oral microbiota and NASH

Microorganisms found in the human oral cavity have been referred to as the oral microflora, oral microbiota, or more recently as the oral microbiome. The oral cavity, or mouth, includes several distinct microbial habitats, such as teeth, gingival sulcus, attached gingiva, tongue, cheek, lip, hard palate, and soft palate. Contiguous with the oral cavity are the tonsils, pharynx, esophagus, Eustachian tube, middle ear, trachea, lungs, nasal passages, and sinuses. Studies demonstrated different oral structures and tissues are colonized by distinct microbial communities [131]. Approximately 280 bacterial species from the oral cavity have been isolated in culture and formally named. It was estimated that less than half of bacterial species present in the oral cavity can be cultivated using anaerobic microbiological methods and that there are likely 500 to 700 common oral species [132]. Cultivation-independent molecular methods, primarily using 16S rRNA gene-based cloning studies, have validated these estimates by identifying approximately 600 species or phylotypes [132]. Microorganisms colonizing one area of the oral cavity have a significant probability of spreading along contiguous epithelial surfaces to neighboring sites. Microorganisms from the oral cavity can cause a number of oral infectious diseases, including periodontitis (gum disease), caries (tooth decay), endodontic (root canal) infections, alveolar osteitis (dry socket), and tonsillitis. Furthermore,

accumulating evidence has linked oral bacteria to a number of systemic diseases [133], including DM [134], cardiovascular disease [135, 136], stroke [137], intracranial hemorrhage [138], preterm birth [139], ulcerative colitis [140], and pneumonia [141]. However, the relationship between oral microbiota and pathogenesis of NASH has not been demonstrated.

Periodontitis and insulin resistance

Periodontal disease is the most common infectious disease affecting tooth-supporting structures. Although a relationship has been reported between infection with periodontal bacteria and the onset of type 2 DM, it is not well recognized in the medical community [142, 143]. In dentistry, understanding the changes in oral microbiota that foretell the early stages of periodontitis and dental caries, prevalent chronic oral diseases, may allow better diagnosis and treatment before the appearance of the characteristic disease manifestations (such as tissue damage in periodontal pockets or dental hard tissue loss). The emergence and evolution of antibiotic resistance in periodontal pathogens has affected the therapeutic success rates for this disease [144, 145]. New approaches are urgently required to control periodontal disease, and microbiome studies offer a promising new angle of attack.

Periodontal inflammation often leads to superficial ulcers on the gingival sulcus, where blood capillaries are exposed to microbial biofilms [146]. Periodontal pathogens are translocated and released from the sulcus into the bloodstream, and clinical trials have demonstrated such transient bacteremia occurring after preventive dental procedures and periodontal therapy, including tooth brushing, chewing, subgingival irrigation, periodontal treatment, and dental extractions, at reported frequencies ranging from 17 to 100 % in infected individuals [147–149]. *Porphyromonas gingivalis* is a major causative agent of periodontitis [150–152]. Recent reports suggest infection with *P. gingivalis* is associated with several systemic diseases, including cardiovascular diseases, preterm low birth weight, rheumatoid arthritis, and DM [150–152]. Moreover, increased serum levels of LPS and TNF-α associated with *P. gingivalis* infection can induce insulin resistance leading to the development of type 2 DM (Fig. 4) [153]. In addition, our colleagues reported a relationship between the fimbrial type of the periodontal bacteria causing periodontitis and the risk of type 2 DM development [154]. Taken together, periodontitis may lead to the development of NASH pathogenesis via exacerbation of insulin resistance.

Periodontitis and NASH

Yoneda et al. demonstrated that the prevalence of *P. gingivalis* infection was significantly higher in NAFLD patients than in healthy subjects. Multiple regression analysis in NAFLD patients and control subjects revealed a significantly higher

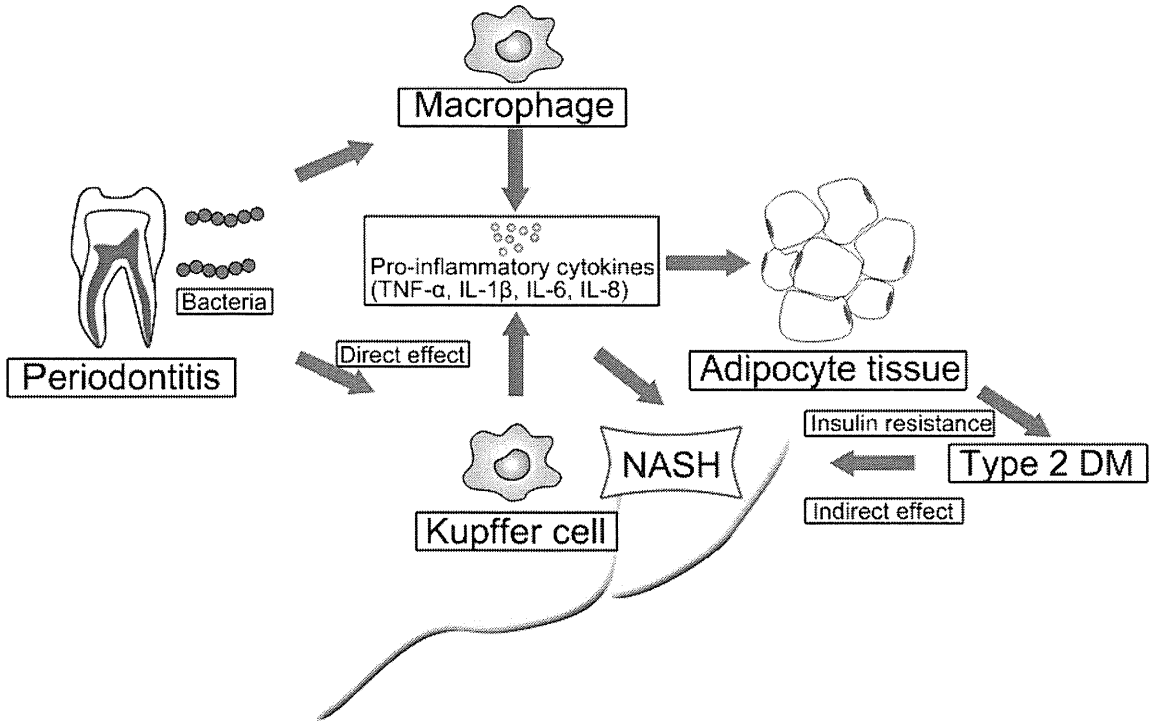

Fig. 4 Proposed mechanisms of periodontitis-induced progression of nonalcoholic steatohepatitis (*NASH*). (*Direct effect*) Periodontal diseases also involve activation of the broad axis of innate immunity through upregulation of pro-inflammatory cytokines from macrophages including Kupffer cells, resulting in liver inflammation. (*Indirect effect*) Increased pro-inflammatory cytokines released into the systemic circulation affect adipose tissue, leading to insulin resistance, which results in the progression of NASH pathogenesis

prevalence of *P. gingivalis* infection in NAFLD patients compared with control subjects, even after adjusting for age, history of DM, and body mass index (BMI). This suggested *P. gingivalis* infection might be involved in the independent risk of onset of NAFLD, because *P. gingivalis* itself or endotoxin and cytokines released from the bacteria can easily enter the blood circulation (Table 1) [155]. These results may indicate that both DM and *P. gingivalis* infection may be involved in the progression of simple steatosis to NASH through direct and/or indirect effects (Fig. 4).

Sterile inflammation and NASH

In addition to infectious insults, sterile tissue injury and cell necrosis can induce profound neutrophilic inflammation. In these conditions, neutrophils do not function as antimicrobial effectors but clear debris and initiate wound healing [156]. Indeed, neutrophils are emerging as central orchestrators of resolution and restitution following tissue injury and may even contribute to the avoidance of autoimmunity following sterile inflammation (SI) [157, 158]. However, excessive or prolonged neutrophil infiltration can exacerbate tissue injury, leading to disease.

The liver is a target of pathogens that induce rapid inflammatory responses via PAMPs [159]. However, less intuitive is that injury in the absence of pathogens can result in inflammation. SI, however, occurs in all tissues after injury of various etiologies. In the liver, SI is particularly important because it is a major component of the pathology of a wide range of diseases, such as alcoholic steatohepatitis (ASH), NASH, drug-induced liver injury, and ischemia/reperfusion [160–162]. Although SI is a well-characterized phenomenon, the mechanisms that initiate and regulate innate immune responses and cell death have only recently begun to be described [163, 164]. The theoretical basis for this originated in the inability of self-recognition and non-self-recognition theories to explain the selectivity of the adaptive immune system. This led to the proposal that danger is used as a criterion for immune activation, and molecules termed "damage-associated molecular patterns" (DAMPs) released from damaged cells are a molecular trigger for inflammation [165]. Liver-resident macrophages (Kupffer cells) and dendritic cells respond to DAMPs by creating an inflammatory milieu that incites the influx and/or activation of T cells, monocytes, and neutrophils, the chief effectors of liver injury (Fig. 3) [166, 167]. This was experimentally validated and explained many aspects of the sterile inflammatory response [166]. Recent advances have identified DAMPs, their receptors, and the cellular machinery required for processing this into a full inflammatory response. Some general concepts have emerged. Firstly, SI is a bona fide inflammatory response, with features including redness, swelling, heat, neutrophilic infiltrate, cytokine production, and tissue damage (Fig. 3). SI is induced in minutes using innate immune pathways but is also present chronically, for example in NASH and ASH. Conceptually, pathogen-driven inflammation and SI are distinct, but functionally, there are many areas of overlap. Many receptors and pathways initially identified as activated by PAMPs are also activated by DAMPs [168, 169]. One example is TLR, which can be activated by LPS as well as cellular high-mobility group protein B1 (HMGB1), a ubiquitously expressed protein that normally binds to the minor groove of nuclear DNA to control the docking and activity of transcription factors by structurally modifying the DNA double helix [170]. By this route, HMGB-1 can increase the activity of cell cycle regulator p53 while negatively affecting transcription of the pro-inflammatory transcription factor NF-κB [170]. During liver injury, HMGB-1 is predominantly released by excessively ROS-generating hepatocytes, which actively secrete HMGB-1 into the circulation without dying [171]. Additionally, in vitro studies have shown that HMGB-1 can be actively released during apoptosis [172] and passively leaks from necrotic cells [173]. Thus, HMGB-1 acts as a proximal danger signal. Due to the unique vascular supply of the liver, PAMPs of intestinal origin and DAMPs of hepatocyte origin collectively contribute to inflammation in a number of diseases. There is also direct interaction with PAMPs, such as LPS stimulating the release of HMGB1 [174].

Probiotics, prebiotics, antibiotics, and periodontitis treatment for NASH

The current standard of care for treating patients with NAFLD and/or NASH focuses on lifestyle interventions, particularly diet and exercise. There is a lack of consensus regarding the most effective and appropriate pharmacologic therapy for NASH, especially without DM, dyslipidemia, hypertension, or obesity. Alterations in intestinal or oral flora and responses to endotoxin may contribute to liver inflammation and the development of NASH, suggesting that reduction of microbiota and endotoxin may improve NASH. Next, we will review the current options of probiotics, prebiotics, antibiotics, and periodontal treatment available for treating NASH.

Probiotics and prebiotics

Probiotics are live microbes that modulate the intestinal microflora and enhance body health. The most common probiotics in the market are *Lactobacilli*, *Streptococci*, and *Bifidobacteria*. Prebiotics are indigestible carbohydrates that stimulate the growth and activity of beneficial bacteria, particularly *Lactobacilli* and *Bifidobacteria*. Some examples