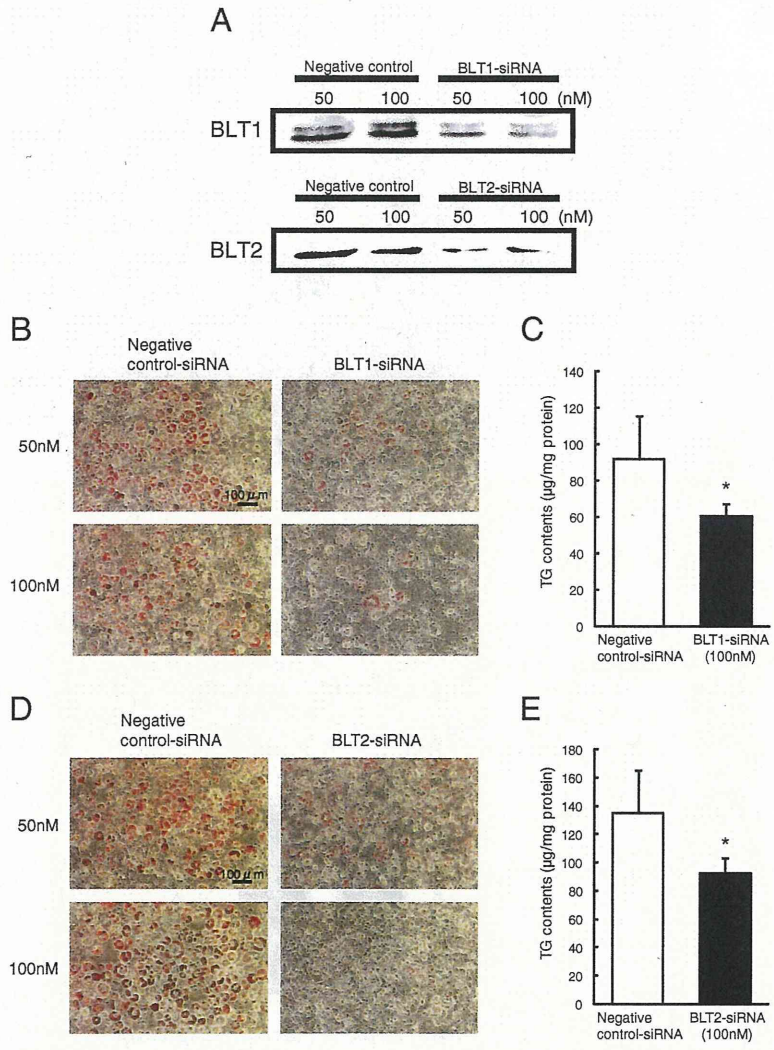

Figure 4 Effect of BLT1 and BLT2 knockdown by siRNA on mouse 3T3-L1 preadipocyte differentiation. (A): Confirmation of BLT1 and BLT2 knockdown by specific siRNAs. Negative control is cells treated with negative control siRNA (Stealth RNAi Negative Control Duplexes). **(B)** and **(C)**: Effect of BLT1 knockdown by siRNA. Representative microscopic **(B)** images of differentiated mouse 3T3-L1 adipocytes by Oil Red O staining. Scale bar represents 100 μm. **(C)**: Accumulation of TG in mature adipocytes was measured and expressed as TG contents (μg/mg protein). Each column represents the mean ± SEM from 3-5 independent experiments. *$P<0.05$ vs. negative control-siRNA treatment. **(D)** and **(E)**: Effect of BLT2 knockdown by siRNA. Representative microscopic **(D)** images of differentiated mouse 3T3-L1 adipocytes by Oil Red O staining. Scale bar represents 100 μm. **(E)**: Accumulation of TG in mature adipocytes was measured and expressed as TG contents (μg/mg protein).

the total volume, and preliminary experiments demonstrated no significant effects of 0.1% DMSO on cell differentiation.

Evaluation of adipocyte differentiation

Differentiation of preadipocytes to mature adipocytes was visually monitored by microscopic observation after Oil red O staining [14,15]. In addition, the amount of triglyceride, an index of lipid accumulation, was quantitatively measured using a Triglyceride E-test Wako kit (Wako Pure Chemicals, Tokyo, Japan). The amount of triglyceride was normalized by protein amount and expressed as TG contents (μg/mg protein).

siRNA for knockdown of BLT1 and BLT2

We designed small interfering RNA (siRNA) for knockdown of BLT1 and BLT2 using an siRNA system (Qiagen, Tokyo, Japan). The sequences of the sense and antisense strand for BLT1 used were 5'-CAACCUACAC UUCCUAUUA-3', and 5'-UAAUAGGAAGUGUAGGU UG-3', respectively. The sequences of the sense and antisense strand for BLT2 used were 5'-GGGACUUA

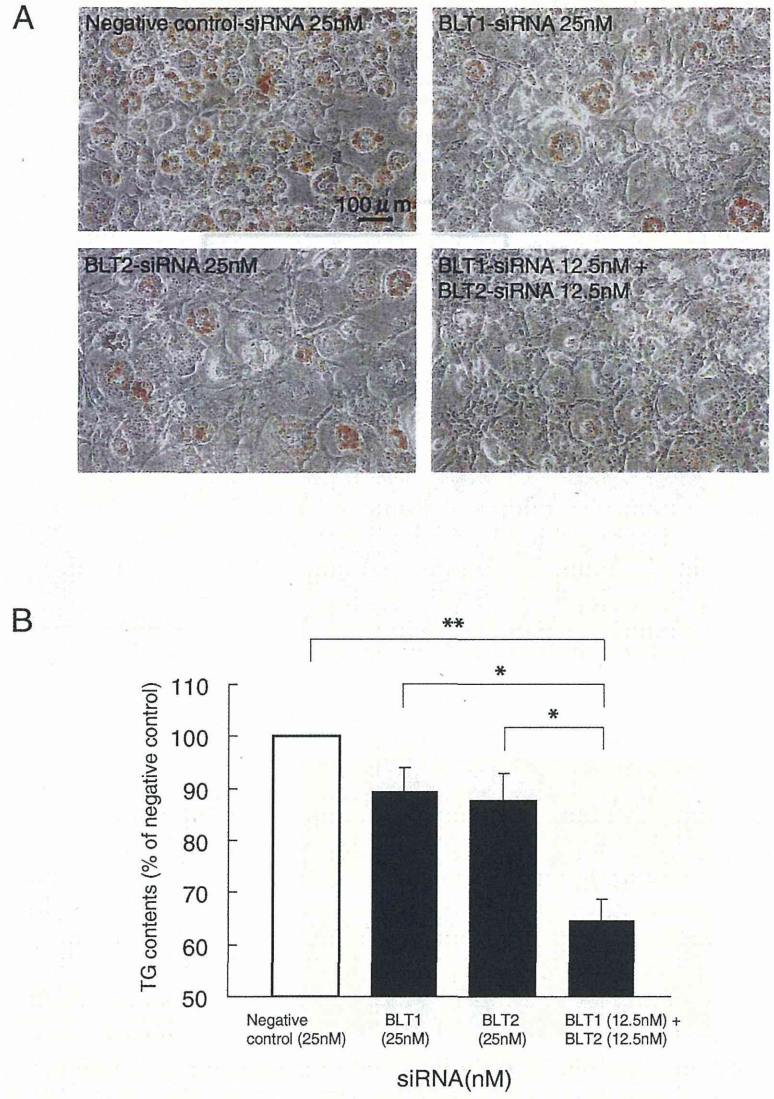

Figure 5 Combination knockdown of BLT1 and BLT2 by siRNA on mouse 3T3-L1 preadipocyte differentiation. Mouse 3T3-L1 preadipocytes were treated with a combination of BLT1-siRNA (12.5 nM) and BLT2-siRNA (12.5 nM). **(A)**: Representative microscopic images of differentiated mouse 3T3-L1 adipocytes by Oil Red O staining. Scale bar represents 100 μm. **(B)**: Accumulation of TG in mature adipocytes was measured and expressed as TG contents (% of negative control). Each column represents the mean ± SEM from 3 independent experiments. *P<0.05, **P<0.01 vs. negative control-siRNA treatment.

ACAUACUCUUA-3', and 5'-UAAGAGUAUGUUAAG UCCG-3', respectively.

For transfection, siRNAs or negative control siRNA (Stealth RNAi Negative Control Duplexes, Invitrogen, Tokyo, Japan) were combined with Lipofectamine RNAiMAX (Invitrogen) and incubated for 20 minutes at room temperature to produce the transfection mixture. Then, the transfection mixture was added to preadipocytes at a final concentration of 25, 50 and 100 nM siRNA (Figure 1). At 24 hours after the start of transfection, the medium was replaced with differentiation medium to induce differentiation. Samples were collected at days 1, 2, 3 and 5 day for western blot analysis, and at day 6 for TG assay and Oil red O staining.

Statistical analysis

Results were expressed as the mean ± SEM. Statistical comparisons were performed using the Student's t-test or Tukey's method after analysis of variance (ANOVA). The results were considered significantly different at P <0.05.

Abbreviations

LT: Leukotriene; LOX: Lipoxygenase; siRNA: Small interfering RNA; TNFα: Tumor necrosis factor alpha; IL-6: Interleukin 6; INS: Insulin;

DEX: Dexamethasone; IBMX: 3-Isobutyl-methylxanthine; ROSI: Rosiglitazone; PPARγ: Peroxisome proliferator-activated receptor gamma; NDGA: Nordihydroguaiaretic acid; TG: Triacylglycerol; PG: Prostaglandin; C/EBPα: CCAAT-enhancer-binding protein, alpha; DMEM: Dulbecco's modified Eagle's medium; DMSO: Dimethyl sulfoxide; ANOVA: Analysis of variance.

Competing interests
The authors declare that they have no competing interests.

Authors' contributions
KH performed all experiments and statistical analysis, discussion of results and drafted the manuscript. KW conceived the study, participated in discussion of the results, provided additional funding for the study. YM assisted in performance of some experiments. AN, YK, TY participated in discussion of the results. All authors read and approved the final manuscript.

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References
1. Fasshauer M, Paschke R: **Regulation of adipocytokines and insulin resistance.** *Diabetologia* 2003, **46**:1594–1603.
2. Yudkin JS: **Inflammation, obesity, and the metabolic syndrome.** *Horm Metab Res* 2007, **39**:707–709.
3. Schinner S, Ulgen F, Papewalis C, Schott M, Woelk A, Vidal-Puig A, Scherbaum WA: **Regulation of insulin secretion, glucokinase gene transcription and beta cell proliferation by adipocyte-derived Wnt signaling molecules.** *Diabetologia* 2008, **51**:147–154.
4. Hillenbrand A, Knippschild U, Weiss M, Schrezenmeier H, Henne-Bruns D, Huber-Lang M, Wolf AM: **Sepsis induced changes of adipocytes and cytokines – septic patients compared to morbidly obese patients.** *BMC Surgery* 2010, **10**:26.
5. Kadowaki T, Hara K, Yamauchi T, Terauchi T, Tobe K, Nagai R: **Molecular mechanism of insulin resistance and obesity.** *Exp Biol Med* 2003, **228**:1111–1117.
6. Samuelsson B, Dahlen SE, Lindgren JA, Rouzer CA, Serhan CN: **Leukotrienes and lipoxins: structures, biosynthesis, and biological effects.** *Science* 2004, **237**:1171–1176.
7. Haeggstrom JZ: **Leukotriene A4 hydrolase/aminopeptidase, the gatekeeper of chemotactic leukotriene B4 biosynthesis.** *J Biol Chem* 2004, **279**:50639–50642.
8. Yokomizo T, Izumi T, Chang K, Takuwa Y, Shimizu T: **A G-protein-coupled receptor for leukotriene B4 that mediates chemotaxis.** *Nature* 1997, **387**:620–624.
9. Brink C, Dahlen SE, Drazen J, Evans JF, Hay DWP, Nicosia S, Serhan CN, Shimizu T, Yokomizo T: **International Union of Pharmacology XXXVII. Nomenclature for leukotriene and lipoxin receptors.** *Pharmacol Rev* 2003, **55**:195–227.
10. Claria J, Lee MH, Serhan CN: **Aspirin-triggered lipoxins (15-epi-LX) are generated by human lung adenocarcinoma cell line (A549)-neutrophil interactions and are potent inhibitors of cell proliferation.** *Mol Med* 1996, **2**:583–596.
11. McMahon B, Mitchell D, Shattock R, Martin F, Brady HR, Godson DC: **Lipoxin, leukotriene, and PDGF receptors cross-talk to regulate mesangial cell proliferation.** *FASEB J* 2002, **16**:1817–1819.
12. Wada K, Arita M, Nakajima A, Katayama K, Kudo C, Kamisaki Y, Serhan CN: **Leukotriene B4 and lipoxin A4 are regulatory signals for neural stem cell proliferation and differentiation.** *FASEB J* 2006, **20**:1785–1792.
13. Tontonoz P, Hu E, Graves RA, Budavari AI, Spiegelman BM: **mPPARgamma 2: tissue-specific regulator of an adipocyte enhancer.** *Genes Dev* 1994, **8**:1224–1234.
14. Katayama K, Wada K, Miyoshi H, Ohashi K, Tachibana M, Furuki R, Mizuguchi H, Hayakawa T, Nakajima A, Kadowaki T, Tsutsumi Y, Nakagawa S, Kamisaki Y, Mayumi T: **RNA interfering approach for clarifying the PPARγ pathway using lentiviral vector expressing short hairpin RNA.** *FEBS Lett* 2004, **560**:178–182.
15. Green H, Kehinde O: **Sublines of mouse 3T3 cells that accumulate lipid.** *Cell* 1974, **1**:113–116.
16. Green H, Meuth M: **An established pre-adipose cell line and its differentiation in culture.** *Cell* 1974, **3**:127–133.
17. Kliewer SA, Lenhard JM, Willson TM, Patel I, Morris DC, Lehmann JM: **A prostaglandin J2 metabolite binds peroxisome proliferator-activated receptor γ and promotes adipocyte differentiation.** *Cell* 1995, **83**:813–819.
18. Evans RM, Barish GD, Wang YX: **PPARs and the complex journey to obesity.** *Nat Med* 2004, **10**:1–7.
19. Spite M, Hellmann J, Tang Y, Mathis SP, Kosuri M, Bhatnagar A, Jala VR, Haribabu B: **Deficiency of the leukotriene B_4 receptor, BLT-1, protects against systemic insulin resistance in diet-induced obesity.** *J Immunol* 2011, **187**:1942–1949.
20. Wada K, Nakajima A, Katayama K, Kudo C, Shibuya A, Kubota N, Terauchi Y, Tachibana M, Miyoshi H, Kamisaki Y, Mayumi T, Kadowaki T, Blumberg RS: **Peroxisome proliferator-activated receptor gamma-mediated neural stem cell proliferation and differentiation.** *J Biol Chem* 2006, **281**:12673–12681.

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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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Keywords GERD · Insomnia · Nonalcoholic fatty liver disease · Proton-pump inhibitor

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

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 ($\times 10^4/\mu$l)	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), $P = 0.8359$].

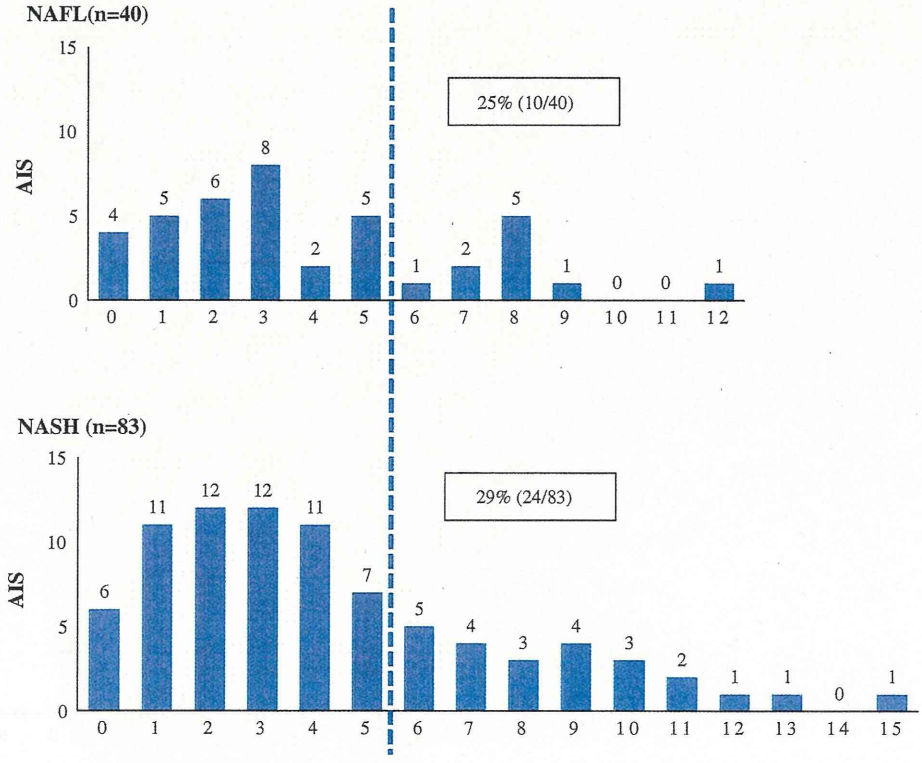

Fig. 1 The distribution of Athens Insomnia scale (AIS)

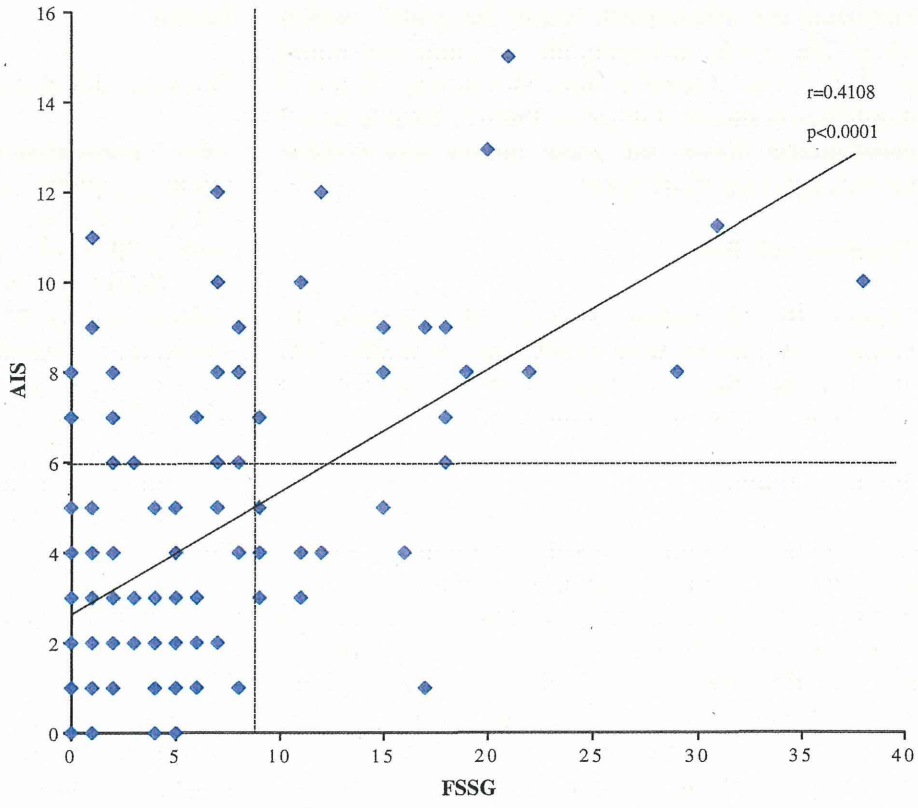

Fig. 2 Correlation between FSSG and AIS. A significant positive correlation was found between AIS and FSSG

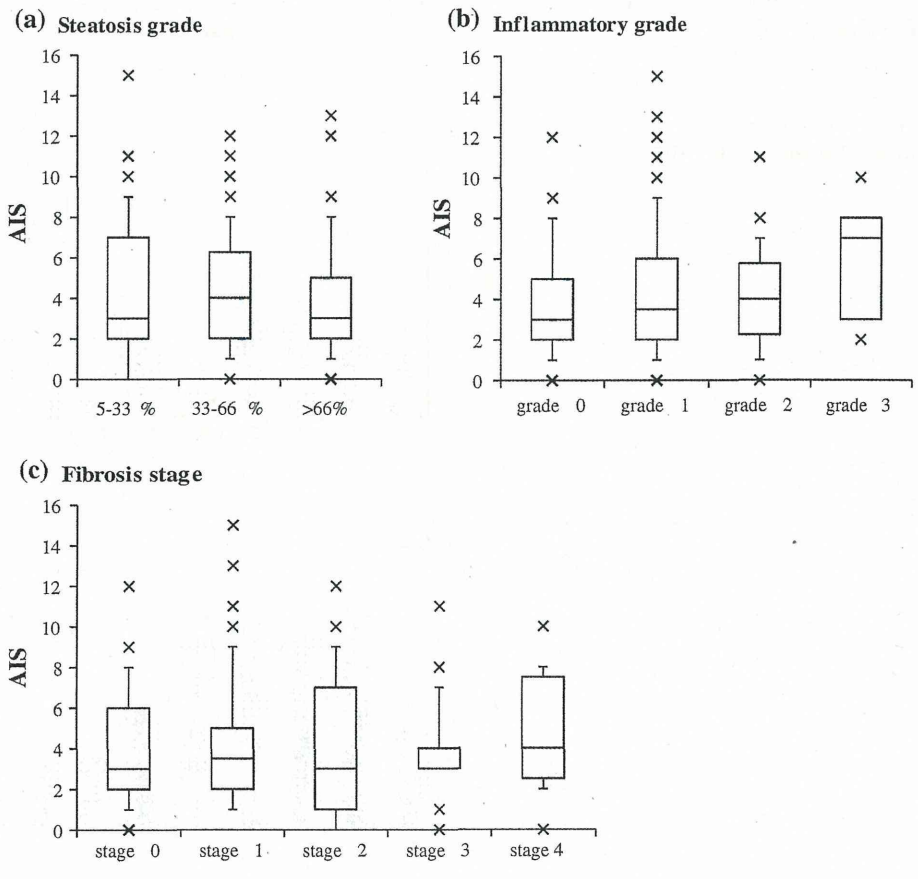

Fig. 3 Relationship between AIS and histological findings. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %

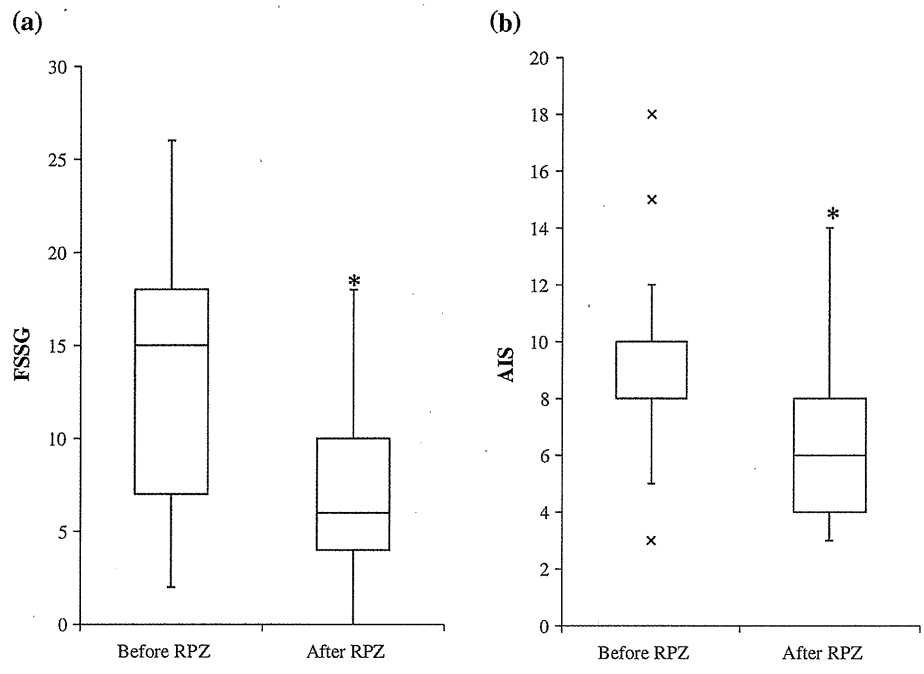

Fig. 4 Effects of rabeprazole (RPZ) on the improvement of GERD symptoms and insomnia. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %. **a** Change in the total FSSG score. RPZ significantly reduced total FSSG scores. $*P = 0.0071$ 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