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/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 $\geq$ 8 (n [%])	31 [25 %]	10 [25 %]	21 [25 %]	1.0000
AIS	3 (0–15)	3 (0–12)	4 (0–15)	0.5591
AIS $\geq$ 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), $P = 0.8359$].

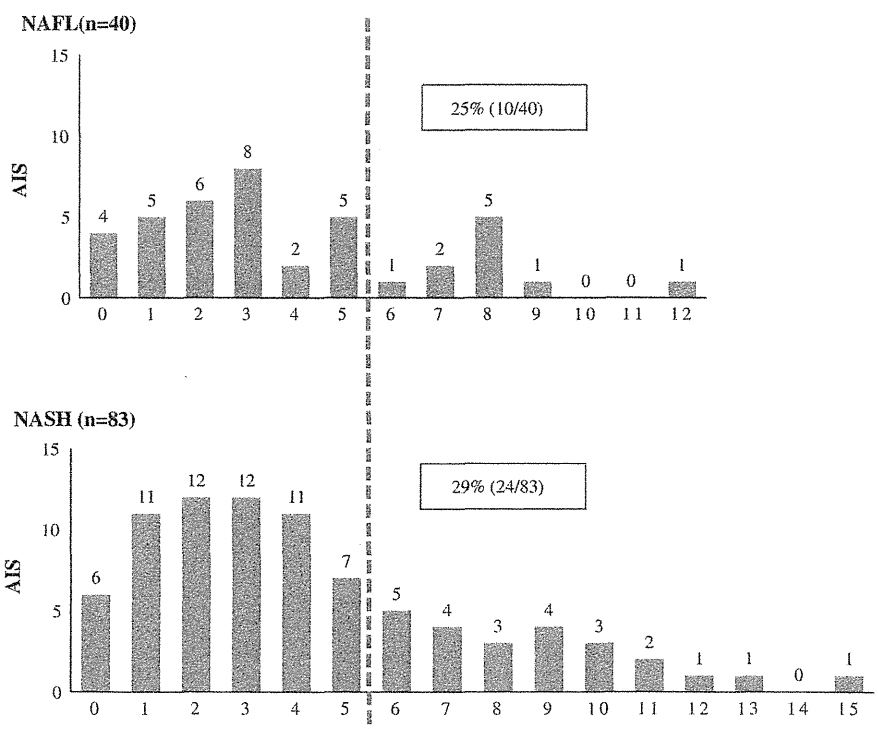

Fig. 1 The distribution of Athens Insomnia scale (AIS)

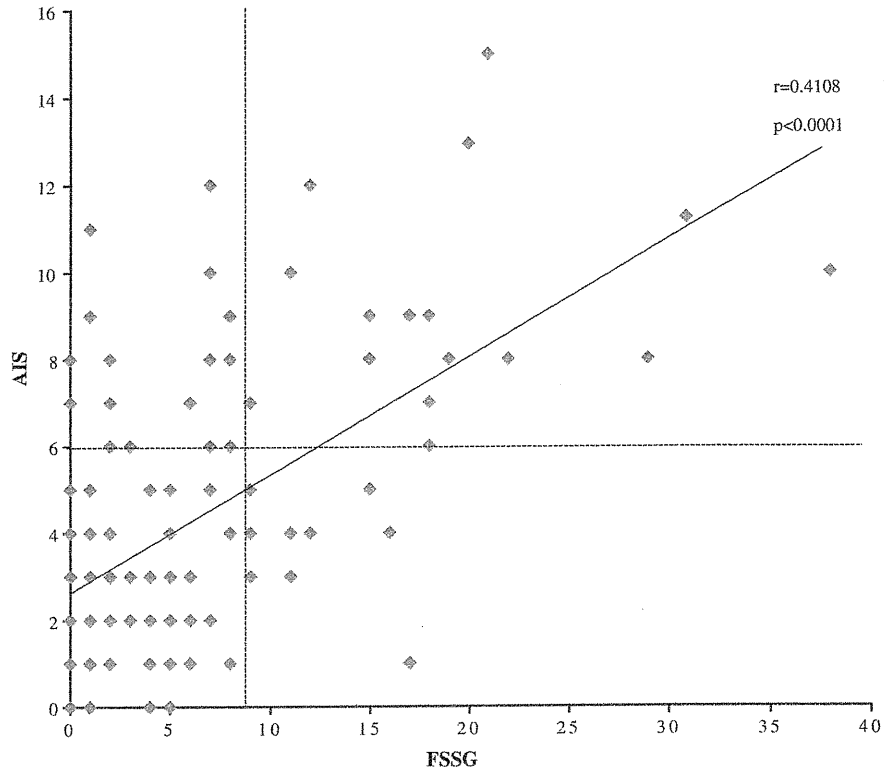

Fig. 2 Correlation between FSSG and AIS. A significant positive correlation was found between AIS and FSSG

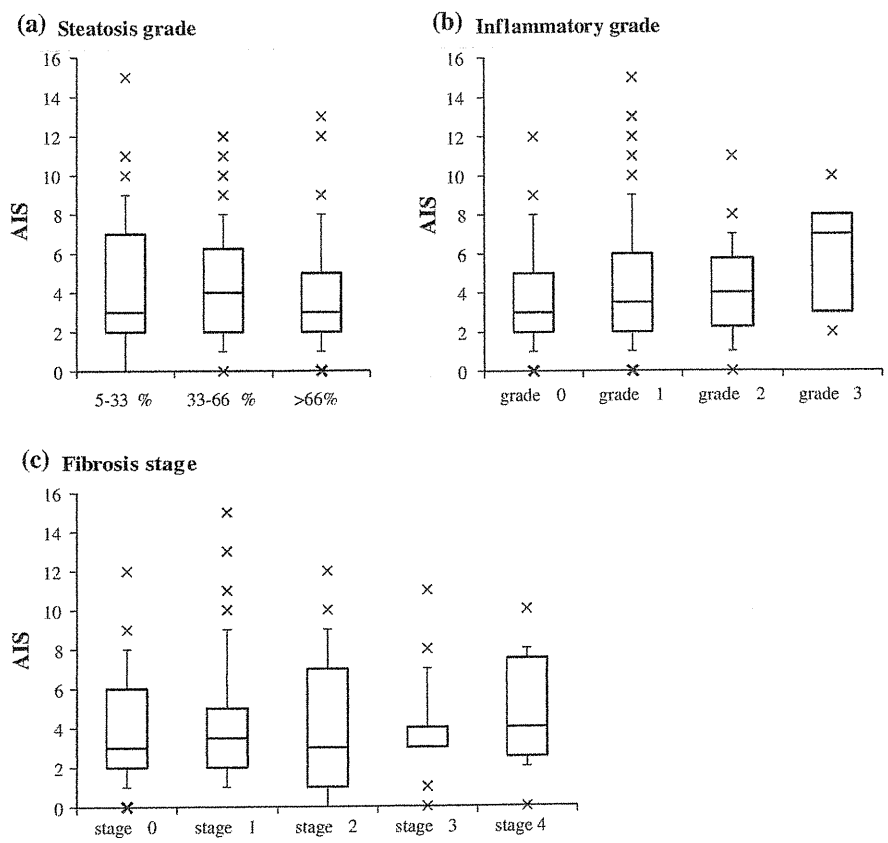

Fig. 3 Relationship between AIS and histological findings. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %

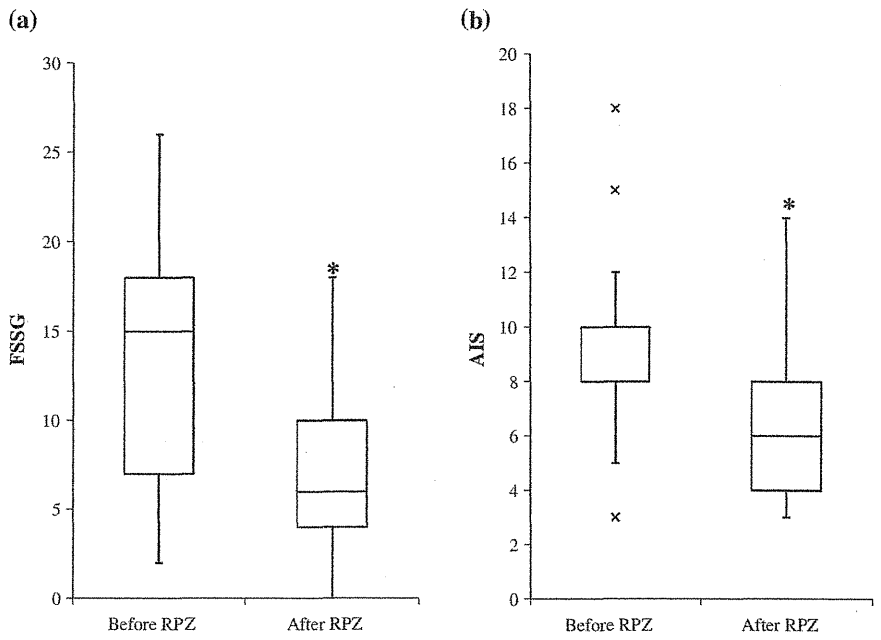

Fig. 4 Effects of rabeprazole (RPZ) on the improvement of GERD symptoms and insomnia. The *box* represents the interquartile ranges (25 and 75 %) from the median (*horizontal line*). The *bars* indicate the 10 and 90 %. **a** Change in the total FSSG score. RPZ significantly reduced total FSSG scores.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

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Glycemic Variability Is an Independent Predictive Factor for Development of Hepatic Fibrosis in Nonalcoholic Fatty Liver Disease

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Abstract

Patients with nonalcoholic fatty liver disease (NAFLD) and nonalcoholic steatohepatitis (NASH) often have metabolic disorders including insulin resistance and type 2 diabetes mellitus (T2DM). We clarified the predictive factors in glucose metabolism for progression of hepatic fibrosis in patients with NAFLD by the 75-g oral glucose tolerance test (75gOGTT) and a continuous glucose monitoring system (CGMS). One hundred sixty-nine patients (68 female and 101 male patients) with biopsy-proven NAFLD with performance with 75gOGTT were enrolled and divided into four groups according to the stage of hepatic fibrosis (F0–3). The proportion of patients with T2DM significantly gradually increased, HbA1c and the homeostasis model assessment of insulin resistance were significantly elevated, and 1,5-anhydroglucitol (1,5-AG) was remarkably decreased with the progression of fibrosis. In the 75gOGTT, both plasma glucose and insulin secretion were remarkably increased with the progression of fibrosis. The only factor significantly associated with advanced fibrosis was 1,5-AG ($P=0.008$) as determined by multivariate logistic regression analysis. We next evaluated the changes in blood glucose during 24 hours by monitoring with the CGMS to confirm the relationship between glycemic variability and progression of fibrosis. Variability of median glucose, standard deviation of median glucose ($P=0.0022$), maximum blood glucose ($P=0.0019$), and ΔMin–max blood glucose ($P=0.0029$) were remarkably higher in severe fibrosis than in mild fibrosis.

Conclusion: Hyperinsulinemia and hyperglycemia, especially glycemic variability, are important predictive factors in glucose impairment for the progression of hepatic fibrosis in NAFLD.

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Introduction

Nonalcoholic fatty liver disease (NAFLD) includes a wide spectrum of liver diseases that range from simple steatosis, which is usually a benign and non-progressive condition, to nonalcoholic steatohepatitis (NASH), which can progress to liver cirrhosis (LC) and hepatocellular carcinoma in the absence of significant alcohol consumption [1–3]. The progression of hepatic fibrosis is an important predictive factor for the development of LC and hepatocellular carcinoma not only in patients with chronic hepatitis C, but also in those with NASH [4]. To inhibit the progression of hepatic fibrosis in NASH, it is important to clarify the predictive factors for progression of hepatic fibrosis.

NASH and NAFLD are considered to be hepatic manifestations of the metabolic syndrome including insulin resistance (IR) and abnormalities of glucose metabolism [5,6]. In accordance with the increased prevalence of obesity and type 2 diabetes mellitus (T2DM) in the general population worldwide, the number of patients with NASH and NAFLD have also increased [7,8]. T2DM is considered to be an independent risk factor for the development of NASH and NAFLD [9,10], and hyperinsulinemia and hyperglycemia are common not only in obese patients, but also in non-obese, non-diabetic patients with NASH [11]. On the other hand, the presence of NASH and NAFLD themselves is also considered to be associated with a high risk of developing T2DM [12].

Postprandial hyperglycemia and glycemic variability were reported to involve progression of atherosclerosis through increase of oxidative stress, activation of inflammatory cytokines and inflammation [13–15]. Oxidative stress is well known as one of most important factor for inflammation and progression of hepatic fibrosis in NAFLD patients [16,17]. The continuous glucose

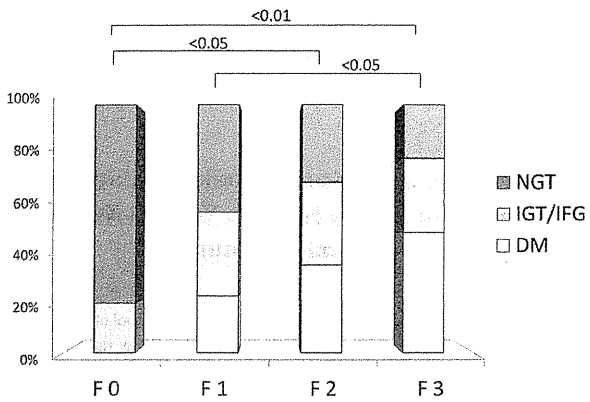

Figure 1. Relationship between glucose impairment and progression of hepatic fibrosis. The frequencies of NGT, IGT/IFG, and T2DM in the four stages of hepatic fibrosis are shown. The diagnosis of glucose impairment was based on the 75gOGTT. The prevalence of NGT in patients in the F0 group (80.0%) was significantly higher than that in the F1 (43.2%), F2 (31.3%), and F3 groups (21.6%), and the frequencies of patients with T2DM in the F3 group (48.6%) was significantly higher than that in the F0 (0%), F1 (22.9%), and F2 (35.4%) groups. *P*-values were calculated using the X^2-test. Fibrosis stage (F): F0 ($n=10$), F1 ($n=74$), F2 ($n=48$), F3 ($n=37$); total, N = 169.
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monitoring system (CGMS) has been introduced as a useful tool, which detect postprandial hyperglycemia [18] and glycemic variability during 24 hours in DM patients. In addition, episodic hypoglycemia during sleeping time can also be detected by CGMS [19]. However, postprandial hyperglycemia and glycemic variability have not yet been evaluated by CGMS in NAFLD patients. Moreover, the relationship between the clinical features of glucose impairment and the progression of hepatic fibrosis in NASH and NAFLD has not been well elucidated. In this study, therefore, we clarified the predictive factors in glucose metabolism for the progression of hepatic fibrosis in NAFLD using the 75-g oral glucose tolerance test (75gOGTT) and CGMS.

Patients and Methods

Patients

A total of 169 patients with biopsy-proven NAFLD (68 female and 101 male patients) with performance with 75gOGTT were enrolled in this study. Liver biopsies had been obtained in all patients after a thorough clinical evaluation had been performed and signed informed consent had been obtained from each patient. Patients with known use of methotrexate, tamoxifen, corticosteroids, or alcohol in excess of 20 g per day and patients with other known causes of liver disease including viral hepatitis, hemochromatosis, Wilson's disease, and autoimmune liver diseases were excluded from this study. None of the patients had received anti-diabetic drugs or insulin. The study protocol conformed to the ethical guidelines of the 1975 Declaration of Helsinki [20] and was approved by the Research Committee of Kochi Medical School.

Clinical and Laboratory Evaluation

Venous blood samples were obtained in the morning after a 12-hour overnight fast. Laboratory tests in all patients included measurements of serum aspartate aminotransferase, alanine aminotransferase, gamma-glutamyl transpeptidase, lipid profiles, total cholesterol, triglycerides, high-density lipoprotein cholesterol, low-density lipoprotein cholesterol, fasting plasma glucose, fasting immunoreactive insulin (f-IRI), creatinine, blood urea nitrogen, 1,5-anhydroglucitol (1,5-AG), HbA1c, and fibrosis markers. These parameters were measured using standard clinical chemistry techniques in the laboratory section of Kochi Medical School Hospital. All patients underwent the 75gOGTT. Plasma glucose and insulin concentrations were measured at 0, 30, 60, 90, 120, and 180 minutes. Insulin resistance was calculated by the homeostasis model (HOMA)-IR using following formula: HOMA-IR = fasting plasma insulin (μU/ml) X fasting plasma glucose (mg/dl)/405. The measure of insulin secretion was calculated by the insulinogenic index using following formula: insulinogenic index = (Δplasma insulin 0–30 min)/(Δplasma glucose 0–30 min).

Histological Evaluation

Liver biopsies of all patients were performed percutaneously under ultrasonographic guidance, and biopsy specimens were obtained from the liver parenchyma of the upper region of the right lobe using a 15-gauge biopsy needle. Liver biopsy specimens were routinely fixed in 10% phosphate-buffered formalin (pH 7.4), embedded in paraffin, and sectioned for hematoxylin and eosin staining. Hepatic fibrosis was assessed by Brunt's classification [21], and fibrosis staging was as follows: 0 = no fibrosis; 1 = zone 3 fibrosis only; 2 = zone 3 and portal/periportal fibrosis; 3 = bridging fibrosis; and 4 = cirrhosis. Histological evaluation was performed by two pathologists with no knowledge of the patients' clinical data.

Continuous Glucose Monitoring System (CGMS)

Continuous glucose levels in 20 patients with biopsy-proven NAFLD were monitored by the CGMS System Gold (Medtronic MiniMed, Northridge, CA, USA). None of the patients had received anti-diabetic drugs, including insulin injection. In the severe hepatic fibrosis group, four patients with NAFLD with F4 fibrosis (LC) were included in this study, unlike in the 75gOGTT study. According to the operating guidelines, the CGMS was installed in the patients to monitor the glucose levels of interstitial fluid [22]. The glucose sensor was inserted into the subcutaneous tissue of the abdomen at 3:00 to 4:00 PM and was monitored for 30 hours. Finger-stick blood glucose levels were checked to calibrate the first glucose value of the CGMS after 1 hour of initialization. Glucose concentrations were determined at least four times per day with an automatic blood glucose meter (Glutest; Sanwa Kagaku Kenkyusho Co., Ltd., Nagoya, Japan). Meals were strictly standardized (1800 kcal/day of standard diets at Kochi Medical School Hospital) during the examination.

Statistical Analyses

Results are presented as mean ± standard deviation for quantitative data and as numbers or percentages for categorical or qualitative data. Statistical differences in quantitative data were determined using the Mann-Whitney U test or post-hoc test. Qualitative data were compared using the chi-square test. Multivariate analysis was carried out by logistic regression. These statistical analyses were carried out using Small Stata 10.1 for Windows. Results were considered significant when the P value was <0.05.

Results

Relationship between glucose impairment and progression of hepatic fibrosis

To investigate the relationship among clinical features of glucose levels, insulin secretion, and hepatic fibrosis, the 169

Table 1. Clinical and physiological characteristics of patients with NAFLD in the four stages of hepatic fibrosis.

	F0 (n=10)			F1 (n=74)			F2 (n=48)			F3 (n=37)		
Gender (F/M)	**4/6**			**25/49**			**18/30**			**21/16**		
Age (yo)	39.6	±	12.2	45.6	±	15.4	49.1	±	14.2	53.6	±	14.3*** ,##
BMI (kg/m^2)	27.4	±	3.0	27.6	±	5.5	28.3	±	4.3	30.1	±	4.6 ##
AST (IU/L)	37.8	±	11.1	43.4	±	20.9	54.5	±	32.8 #	79.3	±	46.1**,###, ++
ALT (IU/L)	64.5	±	29.4	78.2	±	37.1	99.8	±	67.6 #	111.1	±	68.1*, #
ALP (IU/L)	207.2	±	109.9	240.8	±	109.8	287.8	±	100.7	253.1	±	106.3
GGT (IU/L)	113.6	±	101.3	58.2	±	42.9 **	79.7	±	40.0	91.8	±	80.3 #
ChE (IU/L)	375.9	±	59.2	359.8	±	77.7	371.5	±	54.8	328.0	±	82.6
Albumin (g/dl)	4.44	±	0.22	4.58	±	0.28	4.45	±	0.28	4.45	±	0.31
BUN (mg/dl)	13.8	±	2.6	13.8	±	4.0	14.8	±	4.9	13.7	±	3.9
Crn (mg/dl)	0.69	±	0.11	0.84	±	0.97	0.66	±	0.22	0.68	±	0.12
UA (mg/dl)	6.38	±	1.70	6.42	±	2.21	6.38	±	1.29	5.85	±	1.08
FPG (mg/dl)	90.8	±	8.6	104.6	±	24.1	100.6	±	15.5	106.5	±	20.9 *
HbA1c (%)	5.46	±	0.22	5.92	±	1.14	5.96	±	0.77 *	6.27	±	0.98 *
T-Cho (mg/dl)	214.6	±	39.8	203.5	±	35.3	222.2	±	29.8 #	215.3	±	53.7 +
TG (mg/dl)	185.9	±	144.9	150.4	±	90.6	186.8	±	98.0 #	142.7	±	55.0 +
RBC (×10^4/ml)	467.0	±	49.5	478.9	±	49.0	446.4	±	38.9	445.1	±	38.2 #
Hb (g/dl)	14.1	±	1.4	14.3	±	1.8	13.9	±	1.6	13.9	±	0.8
Plt (×10^4/ml)	24.0	±	5.3	23.7	±	5.9	22.8	±	3.8 #	20.0	±	5.4 # ##
WBC (×10^3/ml)	4.47	±	3.18	5.29	±	2.54	5.42	±	2.64	4.02	±	3.10
Fe (mg/dl)	93.2	±	17.6	106.4	±	30.6	107.2	±	20.6	127.1	±	42.6 #
Ferritin (ng/ml)	155.7	±	131.0	241.7	±	172.9	262.9	±	192.6	328.3	±	277.2 #
HA (ng/ml)	20.2	±	13.5	30.4	±	19.5	34.1	±	26.3	93.5	±	105.0 ###, +++
IVcollagen7S (ng/ml)	2.79	±	0.52	3.52	±	0.67 **	3.77	±	1.01 **	5.05	±	2.35*,###, ++
P-3-P (U/ml)	0.53	±	0.14	0.57	±	0.17	0.69	±	0.24	0.92	±	0.66 #

P-values were calculated using the Mann–Whitney U test. Versus F0: *P<0.05, **P<0.01, ***P<0.001. Versus F1: #P<0.05, ##P<0.01, ###P<0.001. Versus F2: ++P<0.01, +++P<0.001. Fibrosis stage (F): F0 (n=10), F1 (n=74), F2 (n=48), F3 (n=37); total, N=169. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; ChE, cholinesterase; UA, uric acid; T-Cho, total cholesterol; TG, triglycerides; FPG, fasting plasma glucose; Plt, platelets; Fe, plasma iron; HA, hyaluronic acid; IV collagen 7S, type IV collagen 7S; P-3-P, type III procollagen N-peptide.

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patients with NAFLD were classified into four groups based on the stage of hepatic fibrosis stage: F0 ($n=10$), F1 ($n=74$), F2 ($n=48$), and F3 ($n=37$). The clinical and physiological data of the four groups are shown in Table 1. The hepatic fibrosis markers hyaluronic acid, type IV collagen 7S, and type III procollagen N-peptide were significantly increased according to the progression of hepatic fibrosis. In addition, the patients with severe fibrosis were much older and had higher ferritin and transaminase levels. The platelet count tended to decrease according to the progression of hepatic fibrosis. We next evaluated the relationship between the prevalence of T2DM diagnosed by 75gOGTT and the stage of hepatic fibrosis in patients with NAFLD. The prevalence of patients with normal glucose tolerance (NGT) was 80%, and no patients with T2DM were found in the F0 group (Fig. 1). On the other hand, the prevalence of NGT in the patients with F3 disease was only 21.6%, and the prevalence of T2DM was 48.6%. In accordance with the progression of hepatic fibrosis, the prevalence of patients with T2DM was significantly gradually increased (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.01$; F1 versus F3, $P<0.05$). To clarify the factors of glucose impairment that are related to the progression of hepatic fibrosis, we evaluated the various parameters of glucose metabolism.

Figure 2A shows that the patients with advanced hepatic fibrosis showed significantly higher levels of HbA1c (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.05$). In addition, 1,5-AG was significantly decreased in accordance with the progression of hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.0001$; F1 versus F3, $P<0.001$; F2 versus F3, $P<0.05$) (Fig. 2B). Severe variability of plasma glucose levels might involve the progression of hepatic fibrosis. HOMA-IR was also elevated in the patients with advanced hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.05$; F1 versus F2, $P<0.05$; F1 versus F3, $P<0.01$) (Fig. 2C). On the other hand, although the insulinogenic index tended to decrease in accordance with the progression of hepatic fibrosis, no statistically significant difference was recognized in our study (Fig. 2D).

We next evaluated the patterns of glucose and insulin secretion by the 75gOGTT in patients with NAFLD. As shown in Figure 3A, not only the fasting glucose levels (F0 versus F3, $P<0.05$), but also the glucose levels after oral glucose loading (at 30, 60, 90, and 120 minutes) were significantly increased in parallel with the progression of fibrosis. The area under the curve (AUC) of the plasma glucose level (AUC-PG) as the marker for total glucose secretion after oral glucose loading also increased in accordance with the progression of hepatic fibrosis (F0 versus F2, $P<0.05$; F0

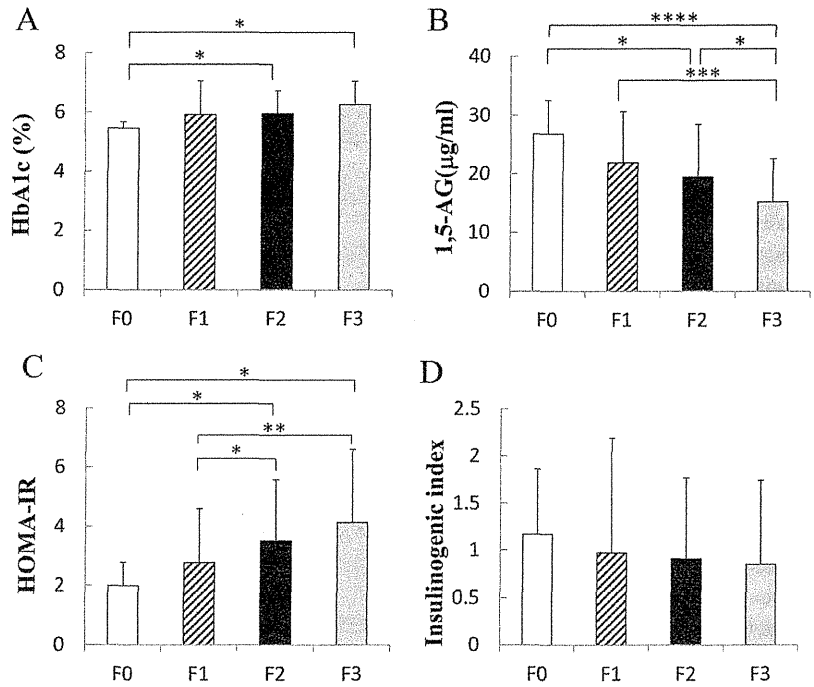

Figure 2. Relationship between hepatic fibrosis and various parameters of glucose metabolism. A) HbA1c was significantly elevated in accordance with the progression of hepatic fibrosis (F0 versus F2, *P<0.05; F0 versus F3, **P<0.05; N = 169). B) 1,5-Anhydroglucitol (1,5-AG) levels were remarkably decreased with the progression of hepatic fibrosis (F0 versus F2, *P<0.05; F0 versus F3, ****P<0.0001; F1 versus F3, ***P<0.001; F2 versus F3, *P<0.05; N = 169). C) HOMA-IR was significantly elevated in the patients with advanced hepatic fibrosis (F0 versus F2, *P<0.05; F0 versus F3, *P<0.05; F1 versus F2, *P<0.05; F1 versus F3, **P<0.01; N = 169). D) The insulinogenic index did not differ among the fibrosis groups (N = 169).
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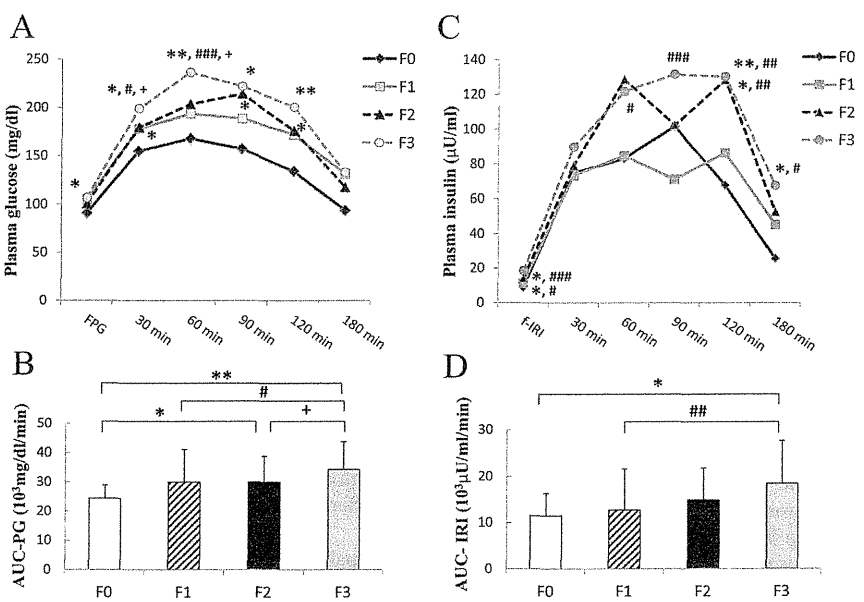

Figure 3. Patterns of glucose and insulin secretion in the 75gOGTT in relation to the progression of hepatic fibrosis. A) The glucose levels were significantly elevated in accordance with the progression of fibrosis (Versus F0: *P<0.05, **P<0.01; Versus F1: $^{\#}P$<0.05, $^{\#\#\#}P$<0.001; Versus F2: ^{+}P<0.05). B) Area under the curve (AUC) of plasma glucose levels (AUC-PG) was remarkably larger in accordance with the progression of hepatic fibrosis (F0 versus F2, *P<0.05; F0 versus F3, **P<0.01; F1 versus F3, $^{\#}P$<0.05; F2 versus F3, ^{+}P<0.05) C) Insulin secretion levels were remarkably higher in the patients with progression of hepatic fibrosis (Versus F0: *P<0.05, **P<0.01; Versus F1: $^{\#}P$<0.05, $^{\#\#}P$<0.01, $^{\#\#\#}P$<0.001). D) AUC of insulin secretion (AUC-IRI) was also significantly larger in accordance with the progression of hepatic fibrosis (F0 versus F3, *P<0.05; F1 versus F3, $^{\#\#}P$<0.01). (Black diamond: F0: n = 10. Gray square: F1: n = 74. Black triangle: F2: n = 48. Gray circle: F3: n = 37; total, N = 169).
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Table 2. Comparison of the parameters of glucose metabolism between patients with mild fibrosis (F0–2) and severe fibrosis (F3).

	F0-2 (n=132)	F3 (n=37)	P value
Gender (F/M)	47/85	21/16	
Age (yo)	46.4 ± 14.9	53.6 ± 14.3	0.00967
BMI (kg/m^2)	27.4 ± 3.0	30.1 ± 4.6	0.01395
FPG (mg/dl)	102.1 ± 20.7	106.5 ± 20.9	0.25665
HbA1c (%)	5.90 ± 0.97	6.27 ± 0.98	0.04520
f-IRI (μU/ml)	12.0 ± 7.5	18.3 ± 11.8	0.00012
HOMA-IR	2.98 ± 1.92	4.14 ± 2.47	0.00320
insulinogenic index	0.96 ± 1.06	0.85 ± 0.89	0.54574
AUC-IRI (10^3μU/ml/min)	12.9 ± 8.3	18.4 ± 9.2	0.00103
AUC-PG (10^3mg/dl/min)	29.6 ± 10.0	34.3 ± 9.5	0.01093
1,5-AG (μg/ml)	21.4 ± 8.8	15.2 ± 7.3	0.00014

P-values were calculated using the Mann–Whitney U test. Data are expressed as mean ± standard deviation. BMI, body mass index; FPG, fasting plasma glucose; f-IRI, fasting immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; AUC-IRI, area under the curve of IRI secretion; AUC-PG, area under the curve of plasma glucose; 1,5-AG, 1,5-anhydroglucitol.
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versus F3, $P<0.01$; F1 versus F3, $P<0.05$; F2 versus F3, $P<0.05$) (Fig. 3B). In addition, f-IRI was significantly elevated in accordance with the progression of hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.05$; F1 versus F2, $P<0.05$; F1 versus F3, $P<0.001$) (Fig. 3C). Furthermore, the AUC of IRI secretion (AUC-IRI) was also significantly increased in accordance with the progression of hepatic fibrosis (F0 versus F3, $P<0.05$; F1 versus F3, $P<0.01$) (Fig. 3D). In particular, the insulin levels at 120 minutes were remarkably higher in the patients with advanced hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.01$; F1 versus F2, $P<0.01$; F1 versus F3, $P<0.01$). On the other hand, insulin secretion levels at 30 minutes were not statistically different among the groups.

To clarify the prognostic factors associated with advanced hepatic fibrosis, the factors that might be related to glucose metabolism were compared between the mild fibrosis group (F0–2) and severe fibrosis group (F3). Table 2 shows that age, body mass index, HbA1c, f-IRI, HOMA-IR, AUC-IRI, and AUC-PG were significantly higher in the F3 group than in the F0–2 group. Furthermore, 1,5-AG was significantly lower in the F3 group than in the F0–2 group ($P=0.00014$). In contrast, fasting plasma glucose and the insulinogenic index were not significantly different between these groups. As determined by multivariate logistic regression analysis, 1,5-AG ($P=0.008$; Z value, -2.65; odds ratio [OR], 0.89509; 95% confidence interval [CI], 0.82473–0.97145) was the only independent factor for association of advanced hepatic fibrosis in patients with NAFLD (Table 3).

Table 3. Factors associated with progression of hepatic fibrosis in multivariate logistic regression analysis.

	Odds ratio	95% CI	Z value	P value
Age (yo)	1.04252	1.00009–1.08676	1.96	0.051
BMI (kg/m^2)	1.08810	0.96010–1.23318	1.32	0.186
HbA1c (%)	0.82385	0.37798–1.79568	−0.49	0.626
f-IRI (μU/ml)	1.15005	0.89106–1.48432	1.07	0.283
HOMA-IR	0.81485	0.32819–2.02311	−0.44	0.659
insulinogenic index	0.59433	0.29955–1.17917	−1.49	0.137
AUC-IRI (10^3μU/ml/min)	1.00006	0.99997–1.00014	1.29	0.196
AUC-PG (10^3mg/dl/min)	1.00000	0.99989–1.00011	−0.08	0.937
1,5-AG (μg/ml)	0.89509	0.82473–0.97145	−2.65	0.008

P-values were calculated using logistic regression. BMI, body mass index; FPG, fasting plasma glucose; f-IRI, fasting immunoreactive insulin; HOMA-IR, homeostasis model assessment of insulin resistance; AUC-IRI: area under the curve of IRI secretion; AUC-PG, area under the curve of plasma glucose; 1,5-AG, 1,5-anhydroglucitol; CI, confidence interval.
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Continuous glucose monitoring system (CGMS) clarified that variability of glucose changes was associated with advanced hepatic fibrosis

In multivariate logistic regression analysis, 1,5-AG was selected as the independent associated factor for advanced hepatic fibrosis. A lower 1,5-AG might indicate not only poor control of plasma glucose, but also severe variability of plasma glucose changes. We hypothesized that severe variability of plasma glucose levels might involve the progression of hepatic fibrosis. To address our hypothesis, we investigated the variability of glucose levels during 24 hours by a CGMS. We used the CGMS for 10 patients in the severe fibrosis group (F3–4), including patients with LC, and 10 patients in the mild hepatic fibrosis group (F0–2). No patients in either group took any anti-diabetes drugs or insulin injections. The clinical data of both groups are shown in Table S1 in File S1.

The average median glucose level of the patients with mild fibrosis (F0–2) was significantly lower than that in the patients with severe fibrosis (F3–4) (108.1±12.1 versus 132.8±39.5 mg/dl, $P<0.00001$) (Fig. 4 and Table 4). The variability of median glucose levels of the patients with mild fibrosis was remarkably smaller than that in the patients with severe fibrosis, as shown in Figure 4. The standard deviation of the median glucose levels in the patients with mild fibrosis was remarkably smaller than that in the patients with severe fibrosis (17.4±5.2 versus 39.7±17.8 mg/dl, $P=0.0022$). In addition, ΔMin–max blood glucose was also significantly larger in patients with severe fibrosis than in those with mild fibrosis (165.0±69.6 versus 115.2±22.8 mg/dl, $P=0.0029$). Furthermore, all postprandial glucose levels (shadowed areas in Fig. 4, from $P<0.05$ to $P<0.001$) and maximum glucose levels ($P=0.0019$) (Table 4) in the patients with severe fibrosis were significantly higher than those in the patients with mild fibrosis, although the minimum blood glucose levels were not significantly different ($P=0.9221$).

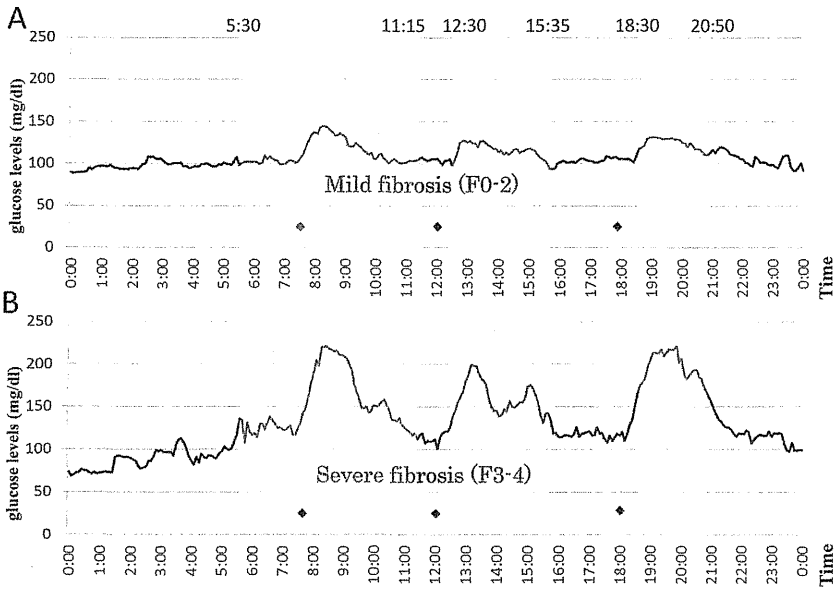

Figure 4. Twenty-four-hour sensor glucose profiles by continuous glucose monitoring system. The changes in the median sensor glucose levels during 24 hours are shown in the patients with A) mild fibrosis (F0–2, $n=10$) and B) severe fibrosis (F3–4, $n=10$). The variability of median glucose levels among the patients with mild fibrosis was remarkably smaller than that among the patients with severe fibrosis. Median glucose levels in the patients with severe fibrosis were higher than those with mild fibrosis (shadows areas, $P<0.05$ to $P<0.001$). Black diamond: Time of meal consumption.
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Discussion

The number of patients with NAFLD and NASH has increased according to the increase in the prevalence of patients with obesity and T2DM worldwide. Patients with NAFLD and NASH often have metabolic disorders including IR and T2DM. In particular, IR is considered to be one of most important background factors for the development of NAFLD and NASH. However, detailed clinical features of impairment of glucose metabolism in patients with NAFLD and NASH are not well understood. In this study, we clarified the predictive factors in glucose metabolism for the development of hepatic fibrosis in patients with NAFLD by the 75gOGTT and CGMS methods.

We evaluated the relationship between the prevalence of T2DM diagnosed by the 75gOGTT and the degree of hepatic fibrosis in patients with NAFLD (Fig. 1). No patients with F0 fibrosis had T2DM, but 80% had NGT. On the other hand, the prevalence of NGT in the patients with F3 fibrosis was only 21.6%, and the prevalence of T2DM was 48.6%. In accordance with the progression of hepatic fibrosis, the prevalence of patients with T2DM was significantly increased and that of NGT was significantly decreased. T2DM is reportedly an independent predictor for the progression of hepatic fibrosis in patients with NAFLD [10]. Our data also indicated that the development of T2DM might induce the development of hepatic fibrosis in patients with NAFLD. On the other hand, however, the presence of NASH and NAFLD themselves is reportedly associated with a high risk of developing T2DM [12].

According to the clinical and physiological data of the four groups of patients with NAFLD (Table 1), age, aspartate aminotransferase, hepatic fibrosis markers, and ferritin were higher and platelets were lower in accordance with the progression of hepatic fibrosis, as previously reported [8,23]. To clarify the

Table 4. Comparison of variable parameters of continuous glucose monitoring between patients with mild fibrosis (F0–2) and severe fibrosis (F3–4).

Variable	Mild fibrosis (F0-2)		Severe fibrosis (F3-4)		P value
Average median blood glucose (mg/dl)	108.1	± 12.2	131.5	± 34.3	<0.00001
Average standard deviation (mg/dl)	17.4	± 5.2	39.7	± 17.8	0.0022
Minimum blood glucose (mg/dl)	81.7	± 28.7	72.5	± 26.4	0.9221
Maximum blood glucose (mg/dl)	118.8	± 12.5	237.5	± 65.1	0.0019
ΔMin–max blood glucose (mg/dl)	115.2	± 22.8	165.0	± 69.6	0.0029

Average median blood glucose: average median glucose of the patients during the 24-hour monitoring period.
Average standard deviation: average standard deviation of blood glucose of the patients during the 24-hour monitoring period.
Minimum and maximum blood glucose values: lowest and highest values, respectively, during the 24-hour monitoring period.
ΔMin–max blood glucose: difference between minimum and maximum blood glucose. Data are expressed as median ± standard deviation.
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detailed glucose impairment related to the progression of hepatic fibrosis, we evaluated the various parameters of glucose metabolism. HbA1c was gradually elevated with the progression of hepatic fibrosis (Fig. 2A). Although HbA1c in the patients with F3 fibrosis was higher than that in the patients with F0 fibrosis, HbA1c in all fibrosis groups was around 6.0% because the glucose impairment in all patients in this study was mild enough to perform the 75gOGTT. On the other hand, 1,5-AG remarkably gradually decreased in accordance with the progression of hepatic fibrosis (Fig. 2B). Considering both the results of HbA1c and those of 1,5-AG, evaluation of 1,5-AG might more closely reflect the glycemic variability in patients with NAFLD, and glycemic variability would be closely related to the progression of hepatic fibrosis. IR is considered to be one of the most important predictive factors for the development of NAFLD and NASH [11]. Therefore, evaluation of HOMA-IR also was investigated in our study. HOMA-IR also gradually increased in accordance with the progression of hepatic fibrosis (Fig. 2C). However, the insulinogenic index, the ability of early insulin secretion, was not significantly different among the groups (Fig. 2D).

We next investigated and evaluated the clinical features of 75gOGTT in patients with NAFLD in relation to the progression of hepatic fibrosis (Fig. 3). After oral glucose loading, glucose levels were increased in the patients with advanced fibrosis (F3) compared with the patients with mild fibrosis (F0–2) (F3 versus F0, $P<0.01$; F3 versus F1, $P<0.001$; F3 versus F2, $P<0.05$ at 60 minutes) (Fig. 3A). The elevation of glucose levels continued until 120 minutes after oral glucose loading in the patients with F3 fibrosis. In addition, the AUC–PG as the marker for total glucose secretion after oral glucose loading also significantly gradually increased in accordance with the progression of hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.01$; F1 versus F, $P<0.05$; F2 versus F3, $P<0.05$) (Fig. 3B). Furthermore, f-IRI was significantly elevated in accordance with the progression of hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.05$; F1 versus F2, $P<0.05$; F1 versus F3, $P<0.001$) (Fig. 3C). These results are agreement with the results in Figure 2C. After oral glucose loading, insulin secretion was relatively quickly elevated in all groups of patients with NAFLD. Insulin secretion levels at 30 minutes were not statistically different among the groups. This result is in agreement with the results of the insulinogenic index (Fig. 2D). The insulin secretion in the mild fibrosis group (F0 and F1) decreased relatively early with the decrease in blood glucose levels. On the other hand, insulin secretion in the advanced fibrosis groups continued until 120 minutes. Therefore, the insulin levels at 120 minutes were remarkably higher in the patients with advanced hepatic fibrosis (F0 versus F2, $P<0.05$; F0 versus F3, $P<0.01$; F1 versus F2, $P<0.01$; F1 versus F3, $P<0.01$), as previously reported [24,25]. Furthermore, the AUC-IRI was also significantly increased in accordance with the progression of hepatic fibrosis (F0 versus F3, $P<0.05$; F1 versus F3, $P<0.01$) (Fig. 3D). It is known that insulin has the potential to function as a growth factor. IR and/or T2DM reportedly may accelerate the progression of NASH through lipogenesis, inflammation, and fibrogenesis [26] and induce cancer growth [27]. Kaji et al. also reported that not only glucose and insulin alone, but also a combination of the two, stimulated the proliferation and activation of hepatic stellate cells. They concluded that the IR status directly accelerates the development of hepatic fibrosis and hepatocarcinogenesis through activation of hepatic stellate cells [28]. Taken together with our results, hyperinsulinemia and hyperglycemia might be related to the progression of hepatic fibrosis in NAFLD.

To elucidate the predictive factors that are associated with the development of hepatic fibrosis in NAFLD, various parameters of glucose metabolism were compared between the mild fibrosis group (F0–2) and severe fibrosis group (F3). In univariate analysis (Table 2), age ($P=0.00967$), body mass index ($P=0.01395$), HbA1c ($P=0.0452$), f-IRI ($P=0.00012$), HOMA-IR ($P=0.0032$), AUC-IRI ($P=0.00103$), and AUC-PG ($P=0.01093$) were significantly higher and 1,5-AG ($P=0.00014$) was significantly lower in the severe fibrosis group than in the mild fibrosis group (Table 1, Figs. 2 and 3). As determined by multivariate logistic regression analysis, only 1,5-AG ($P=0.008$; Z value, −2.65; OR, 0.89509; 95% CI, 0.82473–0.97145) remained as the independently associated factor for advanced fibrosis (Table 3). As mentioned in the above results, it was considered that 1,5-AG might have reflected the glycemic variability in patients with NAFLD in this study.

To confirm the relationship between glycemic variability and progression of hepatic fibrosis in NAFLD, the changes in blood glucose levels during 24 hours were monitored by the CGMS in the patients with NAFLD with severe fibrosis (F3–4, $n=10$) and mild fibrosis (F0–2, $n=10$). CGMS examinations were performed at the inpatient center of Kochi Medical School, and the timing of meals and calories contained in the meals were strictly standardized during the examination. In this study, the severe fibrosis group (F3–4, $n=10$) included three patients with LC (F4) who were all diagnosed with T2DM and whose hyperglycemia was too high to perform the 75gOGTT. However, none of the patients in this study took any anti-diabetic drugs or insulin injections. Figure 4 shows that the variability of the median glucose levels of the patients with mild fibrosis (F0–2) was remarkably smaller than that of the patients with severe fibrosis (F3–4). Furthermore, the standard deviation in severe fibrosis (39.7±17.8 mg/dl) was much larger than that in mild fibrosis (17.4±5.2 mg/dl, $P=0.0022$) (Table 4). Although the minimum blood glucose levels in patients with severe fibrosis tended to be lower than those in patients with mild fibrosis (72.5±26.4 versus 81.7±28.7 mg/dl, $P=0.9221$), the maximum blood glucose level in patients with severe fibrosis was remarkably higher than that in patients with mild fibrosis (237.5±65.1versus 118.8±12.5 mg/dl, $P=0.0019$) (Table 4). As a result, the ΔMin–max blood glucose was also significantly larger in severe fibrosis than in mild fibrosis (165.0±69.6 versus 115.2±22.8 mg/dl, $P=0.0029$). The shadowed areas, which indicate glucose levels, were statistically different between the mild and severe fibrosis groups showed postprandial hyperglycemia, and the hyperglycemias were long continued (Fig. 4). Moreover, we noticed a specific clinical feature of postprandial hyperglycemia in patients with NAFLD. The peaks of postprandial hyperglycemia occurred 1 hour after every meal. Interestingly, glucose levels from midnight to early morning tended to be lower in patients with severe fibrosis and had become elevated by breakfast. The statistical differences in these parameters between the mild and severe fibrosis groups did not change even when three patients with LC (F4) were excluded from the severe fibrosis group (Figure S1 and Tables S2 and S3 in File S1). Moreover, in chronic hepatitis C, even in liver cirrhosis, the changes in blood glucose didn't necessarily show any certain patterns unlike NAFLD (data not shown). Taken together with our results, severe variability of blood glucose changes might be closely related to the progression of hepatic fibrosis in NAFLD.

Postprandial hyperglycemia and glycemic variability are reportedly involved in the progression of atherosclerosis through an increase in oxidative stress, activation of inflammatory cytokines and inflammation [13–15], and induction of other pathogenic complications [29,30,31]. In addition, repetitive postprandial glucose fluctuation reportedly evokes more pronounced adhesion of monocytes to endothelial cells compared with

that induced by stable hyperglycemia [32]. In addition, the main mechanism of monocyte adhesion to endothelial cells has been shown to be increased serum adrenaline induced by postprandial glucose spikes [33]. Furthermore, the importance of glucose variability was recently recognized as an independent factor associated with increasing mortality in patients with diabetes [34,35] and critically ill patients [36,37]. Oxidative stress is well known as one of most important factors for inflammation and progression of hepatic fibrosis in NAFLD [16,17]. Taken together with our results, therefore, variability of blood glucose might also induce monocyte adhesion to endothelial cells, activate inflammatory cytokines and inflammation, and increase oxidative stress in the liver of patients with NAFLD.

There are several limitations of this study. We showed that the prevalence of patients with T2DM was significantly increased (Fig. 1) and age and BMI tended to increase (Table 1) in accordance with the progression of hepatic fibrosis. It is known that age and BMI contribute to both the prevalence of T2DM and the progression of hepatic fibrosis. Therefore, not only progression of T2DM, but also age and BMI, might have influenced the progression of hepatic fibrosis in this study.

In conclusion, we clarified that hyperinsulinemia and hyperglycemia are important predictive factors for the development of hepatic fibrosis in this study. More importantly, variability of blood glucose is one of most important predictive factors in glucose impairment for progression of hepatic fibrosis in NAFLD. Therefore, we might need to reconsider the use of anti-diabetic drugs to inhibit the progression of hepatic fibrosis during treatment of patients with NAFLD.

Supporting Information

File S1 Supplemental Figures and Tables. Figure S1, Twenty-four-hour sensor glucose profiles by continuous glucose monitoring system. The changes in the median sensor glucose levels during 24 hours are shown in the patients with A) F0–2 fibrosis ($n = 10$) and B) F3 fibrosis ($n = 7$). The variability of median glucose levels among the patients with F0–2 fibrosis was remarkably smaller than that among the patients with F3 fibrosis. Median glucose levels in the patients with F3 fibrosis were higher than those with F0–2 fibrosis (shadows areas, $P<0.05$ to $P<0.001$): Time of meal consumption. **Table S1, Comparison of the clinical and physiological characteristics between the patients with mild fibrosis (F0–2) and severe fibrosis (F3–4).** Data are expressed as median ± standard deviation. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; ChE, cholinesterase; T-Cho, total cholesterol; TG, triglycerides; FPG, fasting plasma glucose; Plt, platelets; Fe, plasma iron; HA, hyaluronic acid; IV collagen 7S, type IV collagen 7S; P-3-P, type III procollagen N-peptide. **Table S2, Comparison of the clinical and physiological characteristics between the patients with F0–2 fibrosis and F3 fibrosis.** Data are expressed as median ± standard deviation. BMI, body mass index; AST, aspartate aminotransferase; ALT, alanine aminotransferase; GGT, gamma-glutamyl transpeptidase; ChE, cholinesterase; T-Cho, total cholesterol; TG, triglycerides; FPG, fasting plasma glucose; Plt, platelets; Fe, plasma iron; HA, hyaluronic acid; IV collagen 7S, type IV collagen 7S; P-3-P, type III procollagen N-peptide. **Table S3, Comparison of variable parameters of continuous glucose monitoring between patients with F0–2fibrosis and F3 fibrosis.** Average median blood glucose: average median glucose of the patients during the 24-hour monitoring period. Average standard deviation: average standard deviation of blood glucose of the patients during the 24-hour monitoring period. Minimum and maximum blood glucose values: lowest and highest values, respectively, during the 24-hour monitoring period ΔMin–max blood glucose: difference between minimum and maximum blood glucose. Data are expressed as median ± standard deviation.
(DOCX)

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Author Contributions

Conceived and designed the experiments: MO HH Y. Ikeda KC TS. Performed the experiments: MH MO HH Y. Ikeda RY Y. Ishikawa Y. Nagata K. Munekage TO AH Y. Nozaki-Fujimura NO. Analyzed the data: K. Masuda SN NS. Wrote the paper: MH MO.

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ORIGINAL PAPER

Aromatase-null mice expressing enhanced green fluorescent protein in germ cells provide a model system to assess estrogen-dependent ovulatory responses

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Abstract Enhanced green fluorescent protein (EGFP) has provided us with valuable approaches for tracking living cells. We established a novel line of transgenic mice, which express EGFP in the testis and ovary. Histological analysis demonstrated that spermatids in the testis and oocytes in ovarian follicles beyond preantral stages were positive for EGFP. By exploiting these features, we evaluated ovulatory responses of aromatase-gene (*Cyp19a*) knockout mouse expressing the EGFP transgene, which is totally anovulatory due to 17β-estradiol (E2) deficiency. Ovulation in the knockout mice was induced by sequential injections of E2 on days 1, 4 and 5, pregnant mare serum gonadotropin on day 4 and human chorionic gonadotropin on day 6. Fluorescent oocytes were readily detectable at 15 h after the last gonadotropin injection in the oviduct under a fluorescence stereomicroscope, even when only one oocyte was present. However, when E2 supplementation on day 4 or day 5 in the regimen was omitted, no ovulated oocytes were detected, indicating that exogenous E2 supplementation at the time of gonadotropin stimulation is necessary to induce ovulation in aromatase-gene knockout mice. Our results further demonstrated that the current mouse line can provide an alternative tool to study germ cell biology, including oogenesis, ovulation and senescence.

Keywords Aromatase-knockout mouse · Ovulation · Estrogen · Enhanced green fluorescent protein

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Introduction

Ovulation is a vital step for natural reproductive activity in females. It is well established that the ovulatory process is regulated by various hormones and signaling molecules (Richards 1994; Barnett et al. 2006; Drummond and Fuller 2012). We noted during ovulatory stimulation experiments that oocytes released from the ovary were not always localized in the ampulla of the oviduct. Furthermore, inaccurate estimations of ovulatory efficacy might occur when genetically manipulated mice with ovulatory impairment were employed in studies. An aromatase-deficient mouse, in which estrogen synthesis is impaired (Fisher et al. 1998; Toda et al.

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