Table 1 The distribution of histological fibrosis stage in serum ALT levels

ALT range	Patients no.	Stage 0	Stage 1	Stage 2	Stage 3	Stage 4	Advanced fibrosis ratio (%)
All patients	1,102	240 (21.8 %)	339 (30.8 %)	290 (26.3 %)	196 (17.8 %)	46 (4.2 %)	22.0
≤40	235	91 (38.7 %)	65 (27.7 %)	41 (17.4 %)	21 (8.9 %)	17 (7.2 %)	16.1
41–60	229	48 (21.0 %)	76 (33.2 %)	49 (21.4 %)	41 (17.9 %)	15 (6.6 %)	24.5
61–80	172	35 (20.3 %)	53 (30.8 %)	56 (32.6 %)	23 (13.4 %)	5 (2.9 %)	16.2
81–100	122	18 (14.8 %)	41 (33.6 %)	38 (31.1 %)	31 (25.4 %)	3 (2.5 %)	27.9
≥101	344	48 (14.0 %)	104 (30.2 %)	106 (30.8 %)	80 (23.3 %)	6 (1.7 %)	25.0

significantly lower than that among the NAFLD patients with serum ALT levels >40 IU/l, as evaluated by Fisher's exact test ($p = 0.0163$).

The demographic and laboratory characteristics of NAFLD patients with serum ALT levels ≤40 IU/l and the clinical and laboratory features of the subjects with no or mild fibrosis (stage 0–2) compared with those of the patients with advanced fibrosis (stage 3–4) are shown in Table 2. In the patient group with serum ALT levels ≤40 IU/l, comparison of the characteristics of the subjects with no or mild fibrosis with those of subjects with advanced fibrosis revealed significantly higher values of age, serum AST, serum HDL cholesterol, fasting plasma glucose, serum fasting IRI, HOMA-IR, serum hyaluronic acid and serum type IV collagen 7s domain, and lower values of serum cholinesterase, serum albumin, hemoglobin, and platelet count in subjects with advanced fibrosis. In addition, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR were all significantly higher in patients with advanced fibrosis as compared with the values in the patients with no or mild fibrosis in the patient group with serum ALT levels ≤40 IU/l (Table 2).

The AUROC of the platelet count and serum level of the type IV collagen 7s domain for detecting cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l

Because significant differences in the platelet count and type IV collagen 7s domain were observed between patients with advanced fibrosis and those with no or mild fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l, the AUROCs of the platelet count and serum level of type IV collagen 7s domain for detecting fibrosis stages ≥ stage 3 were calculated. The AUROC for estimating the diagnostic performance of the platelet count for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.786 (optimal cutoff value 19.3 × 10^4/μl, sensitivity 81.6 %, specificity 65.5 %) (Fig. 1a). The AUROC for estimating the diagnostic performance of the serum level of type IV collagen 7s for hepatic fibrosis stages ≥ stage 3 among NAFLD patients with serum ALT levels ≤40 IU/l was 0.794 (optimal cutoff value 5.0 ng/ml, sensitivity 63.2 %, specificity 84.3 %) (Fig. 1b).

The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

In order to investigate the diagnostic accuracy of the scoring systems in NALFD with various serum ALT levels, the AUROC for detecting fibrosis stages ≥ stage 3 was calculated in various distributions of serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) (Table 3). The AUROCs were calculated for the FIB-4 index (0.706–0.878), NAFLD fibrosis score (0.657–0.843), BARD score (0.517–0.684), and AAR (0.684–0.804). The diagnostic accuracy of each scoring system was more than equivalent also in case of ALT ≤40 IU/l. Furthermore, concerning the AUROC of the FIB-4 index, the NAFLD fibrosis score had the highest value in case of ALT ≤40 IU/l.

In NAFLD patients in the ALT >40 group, the optimal cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were 1.499, 0.502, 2, and 0.723, which were close to the cutoff values reported before [19, 20, 31, 32].

Prediction of the presence of advanced liver fibrosis and resetting of the cutoff value in NAFLD with normal ALT levels

In order to investigate the diagnostic accuracy of the scoring systems, ROC curves were constructed (Fig. 2). Then, the AUROC, optimal cutoff value, sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) were determined for each of the scoring systems. The FIB-4 index ranged from 0.305 to 12.482 in the NAFLD group with serum ALT values ≤40 IU/l. The FIB-4 index values stratified by the fibrosis stage were as follows: stage 0, 1.233 ± 0.711; stage 1, 1.570 ± 1.167; stage 2, 1.870 ± 1.496; stage 3, 3.071 ± 1.703; stage 4, 6.010 ± 3.704. Thus, in the NAFLD group with serum ALT values ≤40 IU/l, the FIB-4 index increased with increasing histological severity of the

Table 2 Characteristics of the NAFLD patients with serum ALT values ≤40 IU/l

Variable	Total ($n = 235$)	No or mild fibrosis (stage 0–2)	Advanced fibrosis (stage 3, 4)	p value
Age (years)	59.9 ± 12.1	58.6 ± 11.3	66.7 ± 8.4	0.0112
Body mass index (kg/m^2)	26.9 ± 4.0	26.5 ± 4.0	28.7 ± 4.7	0.0564
AST (IU/l)	24.7 ± 10.2	23.3 ± 6.93	31.9 ± 8.60	<0.001
ALT (IU/l)	23.7 ± 7.0	23.8 ± 4.56	23.0 ± 5.38	0.5293
Alkaline phosphatase (IU/l)	271.9 ± 124.4	264.3 ± 123.3	311.3 ± 205.3	0.2206
GGT (IU/l)	52.7 ± 74.1	51.9 ± 43.3	56.9 ± 54.1	0.6927
Cholinesterase (IU/l)	345.6 ± 90.6	366.3 ± 91.0	238.1 ± 104.4	<0.001
Albumin (g/dl)	4.28 ± 0.79	4.39 ± 1.06	3.71 ± 0.45	0.0177
Total cholesterol (mg/dl)	202.6 ± 37.9	203.5 ± 34.9	198.1 ± 43.7	0.6152
LDL cholesterol (mg/dl)	124.7 ± 31.3	127.1 ± 29.2	112.5 ± 31.6	0.1426
HDL cholesterol (mg/dl)	53.9 ± 13.5	52.4 ± 13.3	61.9 ± 16.2	0.0300
Triglyceride (mg/dl)	140.4 ± 72.2	146.2 ± 74.8	110.1 ± 60.8	0.0934
FPG (mg/dl)	123.3 ± 48.2	117.7 ± 40.1	152.1 ± 93.0	0.0176
Fasting insulin (μU/ml)	11.4 ± 8.31	10.6 ± 9.00	15.7 ± 9.01	0.0901
HOMA-IR	3.96 ± 3.56	3.39 ± 3.63	6.96 ± 7.77	0.0127
HbA1c (%)	5.96 ± 0.94	6.07 ± 0.80	5.42 ± 0.78	0.0885
Hemoglobin (g/dl)	13.5 ± 1.61	13.7 ± 1.38	12.2 ± 2.51	0.0010
Platelet count (×10^4/μl)	21.1 ± 6.90	22.7 ± 6.56	12.6 ± 6.18	<0.001
Hyaluronic acid (ng/ml)	87.3 ± 119.3	76.9 ± 128.9	141.0 ± 86.3	0.1302
Type IV collagen 7s (ng/ml)	4.74 ± 1.65	4.29 ± 1.38	7.05 ± 2.26	<0.001
Dyslipidemia	150 (63.8 %)	132 (67.0 %)	18 (47.3 %)	–
Diabetes mellitus	108 (46.0 %)	88 (44.7 %)	20 (52.6 %)	–
Steatosis grade (1/2/3)	124/84/27	94/79/24	30/5/3	–
Inflammatory grade (0/1/2/3)	59/138/38/0	57/117/23/0	2/21/15/0	
Fibrosis stage (0/1/2/3/4)	91/65/41/21/17	–	–	–
FIB 4 index	2.03 ± 1.93	1.47 ± 1.09	4.92 ± 3.42	<0.001
AST/ALT	1.07 ± 0.34	1.01 ± 0.32	1.41 ± 0.30	<0.001
NAFLD fibrosis score	−0.69 ± 1.81	−1.18 ± 1.63	1.82 ± 1.41	<0.001
BARD score	2.46 ± 1.23	2.28 ± 1.09	3.39 ± 0.65	0.0006

Values are mean ± SD. p values from Student's t test, Mann-Whitney test or χ^2 test, as appropriate

AST aspartate aminotransferase, *ALT* alanine aminotransferase, *GGT* γ-glutamyl transpeptidase, *FPG* fasting plasma glucose, *HOMA-IR* homeostasis model assessment-insulin resistance

hepatic fibrosis ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the FIB-4 index for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT ≤40 IU/l was 0.878 (Fig. 2a) (optimal cutoff value 1.659, sensitivity 89.5 %, specificity 71.1 %). Using the previously published cutoff value proposed by Shah et al. [31] (>2.67), the sensitivity of this index for the detection of advanced fibrosis was calculated as 63.2 % and the specificity as 88.3 % (Table 4).

The NAFLD fibrosis score ranged from −6.304 to 4.639 in the NAFLD patients with serum ALT values ≤40 IU/l. The NAFLD fibrosis scores stratified by the fibrosis stage were as follows: stage 0, −1.439 ± 1.538; stage 1, −1.290 ± 1.592; stage 2, −0.762 ± 1.591; stage 3, 0.256 ± 1.400; stage 4, 2.110 ± 1.332; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the NAFLD fibrosis score for hepatic fibrosis stages ≥ stage 3 in the NAFLD patients with serum ALT values ≤40 IU/l was 0.843 (Fig. 2b) (optimal cutoff value 0.735, sensitivity 68.4 %, specificity 88.3 %). Using the previously published cutoff point proposed by Angulo et al. [19] (>0.676), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 68.4 % and the specificity as 87.8 % (Table 4).

The BARD score ranged from 0 to 4 in the NAFLD patients with serum ALT values ≤40 IU/l. The BARD scores stratified by the fibrosis stage were as follows: stage 0, 2.000 ± 1.200; stage 1, 1.967 ± 1.303; stage 2, 2.100 ± 1.215; stage 3, 2.316 ± 1.057; stage 4, 3.333 ± 0.724. The AUROC calculated to estimate the

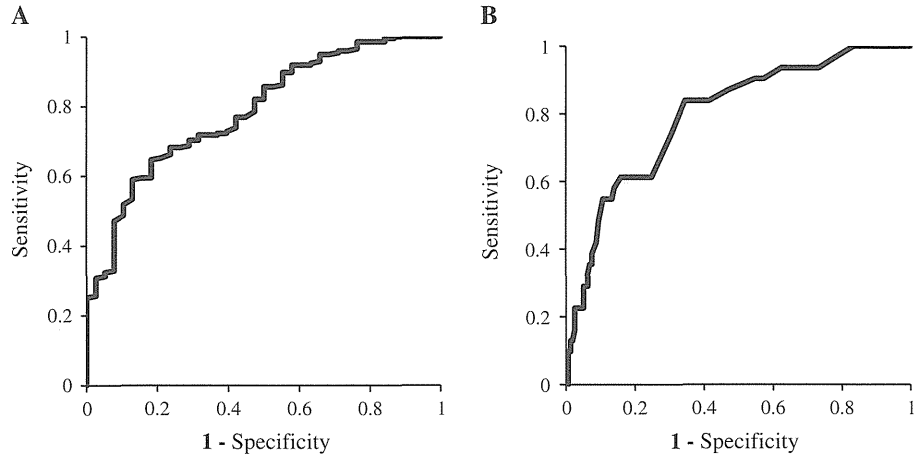

Fig. 1 Receiver-operating characteristic (ROC) curves for detecting advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** The platelet count, **b** type IV collagen 7s

Table 3 The AUROC of each scoring system for detecting advanced fibrosis in various distributions of serum ALT levels

ALT levels (IU/l)	FIB4 index	NAFLD fibrosis score	BARD score	AST/ ALT
≤40	0.878	0.843	0.671	0.794
41–60	0.818	0.726	0.684	0.804
61–80	0.706	0.654	0.663	0.737
81–100	0.752	0.657	0.517	0.684
≥101	0.773	0.670	0.578	0.728

diagnostic performance of the BARD score for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.671 (Fig. 2c) (optimal cutoff value 3, sensitivity 65.8 %, specificity 59.9 %). Using the previously published cutoff point proposed by Harrison et al. [20] (>2), the sensitivity of this scoring system for the detection of advanced fibrosis was calculated as 86.8 % and the specificity as 32.5 % (Table 4).

AAR ranged from 0.448 to 2.630 in the NAFLD patients with serum ALT values ≤40 IU/l. The AAR values stratified by the fibrosis stage were as follows: stage 0, 0.869 ± 0.221; stage 1, 0.942 ± 0.269; stage 2, 0.998 ± 0.424; stage 3, 1.232 ± 0.423; stage 4, 1.359 ± 0.361; thus, the NAFLD fibrosis score increased with increasing histological severity of hepatic fibrosis in this patient group ($p < 0.0001$). The AUROC calculated to estimate the diagnostic performance of the AAR for hepatic fibrosis stages ≥ stage 3 in NAFLD patients with serum ALT values ≤40 IU/l was 0.794 (Fig. 2d) (optimal cutoff value 0.975, sensitivity 78.9 %, specificity 70.1 %). Using the previously published cutoff point proposed by McPherson et al. [32] (>0.8), the sensitivity of this ratio for the detection of advanced fibrosis was calculated as 89.5 % and the specificity as 37.1 % (Table 4).

Discussion

The incidence of NAFLD is rising rapidly in both adults and children because of the currently ongoing epidemics of obesity and type 2 diabetes [33]. Thus, development of a rapid and non-invasive method for the detection of fibrosis in NAFLD patients is of major clinical interest. In recent years, Shah et al. [31] reported, from a multicenter trial, the usefulness of scoring systems for NAFLD patients. In their study, they evaluated 541 NAFLD patients and concluded that the AUROC values calculated to estimate the diagnostic performances of FIB4, the NAFLD fibrosis score, and AAR in which the serum ALT is included for hepatic fibrosis stages ≥ stage 3 were 0.802, 0.768, and 0.720, respectively. We also previously validated these scoring systems in 576 biopsy-proven Japanese NAFLD patients [34]. Furthermore, in this study, the cutoff values of the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR for the diagnosis of advanced fibrosis were close to the cutoff values reported before [19, 20, 31, 32].

NAFLD often presents as abnormal liver enzyme levels in the absence of markers of other common liver diseases, e.g., hepatitis C. The severity of hepatic fibrosis tends to be underestimated in patients with serum ALT values within normal limits, even though normal serum ALT values do not guarantee the absence of advanced fibrosis in patients with NAFLD [23]. It is not uncommon for patients to present with complications of previously unrecognized cirrhosis despite being under long-standing medical care, because these patients often do not manifest the classical physical changes associated with cirrhosis. At present,

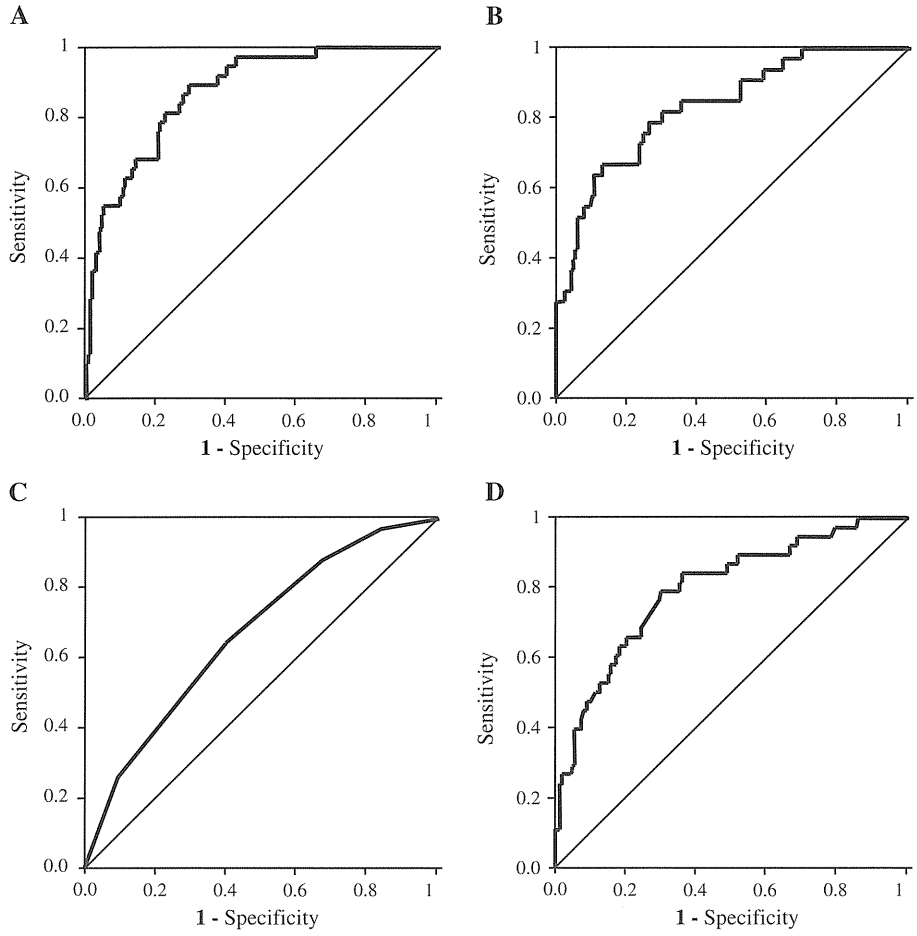

Fig. 2 Receiver-operating characteristic (ROC) curves for the noninvasive scores for a diagnosis of advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** FIB-4 index, **b** NAFLD fibrosis score, **c** BARD score, **d** AST/ALT ratio (AAR)

Table 4 Comparison of the performance of each of the scoring systems for the diagnosis of advanced fibrosis in 235 NAFLD patients with serum ALT values under 40 IU/l using reported cutoff and reset cutoff values

	Cutoff value	Sensitivity (%)	Specificity (%)	PPV (%)	NPV (%)	References
Fib-4						
Reported by Shah et al.	2.67	63.2	88.3	51.1	92.6	[31]
Re-setup	1.659	89.5	71.1	37.4	97.2	
NAFLD fibrosis score						
Reported by Angluo et al.	0.676	68.4	87.8	52.0	93.5	[19]
Re-setup	0.735	68.4	88.3	53.0	93.5	
BARD score						
Reported by Harrison et al.	2	86.8	32.5	19.9	92.8	[20]
Re-setup	3	65.8	59.9	24.0	90.1	
AST/ALT (AAR)						
Reported by McPherson et al.	0.8	89.5	37.1	21.5	94.8	[32]
Re-setup	0.975	78.9	70.1	30.7	94.5	

PPV positive predictive value, *NPV* negative predictive value

NAFLD patients with normal serum ALT values are very rarely investigated or subjected to liver biopsy. Mofrad et al. [23] and Fracanzani et al. [25] found that the histological features of NAFLD sometimes progress even in persons with normal serum ALT values and that the liver histology in these persons is not very different from that in

patients with high serum ALT levels; in addition, a low or normal serum ALT level does not serve as a reliable criterion to exclude the need for liver biopsy in NAFLD patients [23, 25]. Fracanzani et al. [25] reported that a persistent increase of the serum ferritin level, persistent evidence of severe steatosis on ultrasonography, and a persistent increase of the serum GGT levels were the main reasons for liver biopsy in patients with normal serum ALT levels. Mofrad et al. [23] reported that the principal indications for liver biopsy in patients with normal ALT levels were persistent hepatomegaly, donor evaluation for living donor liver transplantation, elevated serum ferritin levels, abnormal imaging characteristics of the liver suggestive of parenchymal liver disease, baseline biopsy to initiation of methotrexate therapy, and clinical features of portal hypertension without other evidence of liver disease.

A first finding in our study is the ratio of advanced fibrosis (stage 3–4) in various distributions of ALT. Advanced fibrosis was seen in 16.1 % of subjects with serum ALT levels ≤40 IU/l. Thus, caution must be exercised in evaluating the disease severity in NAFLD patients with normal serum ALT values. While the platelet count and serum level of the collagen 7s domain were reported to be useful for predicting the presence of advanced fibrosis in NAFLD patients [35, 36], it appears that they may also be useful for predicting severe fibrosis in cases of NAFLD with normal serum ALT levels. However, the specificity of the platelet count and sensitivity of type IV collagen 7s were slightly low. So far, no previous studies have investigated the usefulness of the available tests for the prediction of liver fibrosis in NAFLD patients with normal serum ALT values, because the small sample size of NAFLD subjects with normal serum ALT levels hampers any attempt to construct scoring systems for predicting NASH or fibrosis [25]. Thus, the previous scoring systems, especially their cutoff values, seem to be insufficient for the diagnosis of fibrosis in the NAFLD patients with normal ALT.

A second finding of this study was that the scoring systems investigated in NALFD with normal ALT. Of these, especially the FIB-4 index and NAFLD fibrosis score were clinically very useful (AUROC >0.8) even in patients with normal serum ALT values. Furthermore, with resetting of the cutoff values, they were found to have a higher sensitivity and higher specificity for the prediction of advanced fibrosis in a retrospective cohort of NAFLD patients with normal serum ALT values. The BARD score failed to detect the outstanding sensitivity and specificity in all the ALT groups. Consistent with the present study, Fujii and colleagues [37] reported significantly poorer applicability of the BARD score in Japanese patients with NAFLD compared to Caucasian subjects. It has been suggested that the BARD score is less predictive of advanced fibrosis in Japanese NAFLD patients because they are less obese that those in western countries.

As a third finding of this study, the FIB-4 index, NAFLD fibrosis score, BARD score, and AAR all had high NPVs (>90.1 %) for advanced fibrosis in the cohort of patients with NAFLD. This suggests that these scoring systems could be used clinically to exclude advanced fibrosis in subjects with NAFLD. For example, using the FIB-4 index (<1.659) to exclude advanced fibrosis, liver biopsy could have been avoided in 60.4 % of the patients in our cohort of patients with serum ALT values ≤40 IU/l. Similarly, prediction of the presence/absence of fibrosis based on the NAFLD fibrosis score (<0.735), BARD score (<3), and AAR allowed avoidance of liver biopsy in 66.4, 51.9, and 62.1 % of patients, respectively. Given the large numbers of NAFLD patients with normal serum ALT values, use of these non-invasive tests with reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

As a fourth finding of this study, in contrast to the NPVs, the PPVs of the tests did not have sufficient accuracy for the diagnosis of advanced fibrosis. It would, therefore, seem appropriate to consider liver biopsy in all patients with values above the cutoff of the selected index. We previously reported, for the first time in the world, that transient elastography and acoustic radiation force impulse (ARFI) elastography can be used to measure the severity of fibrosis in patients with NAFLD [15, 38]. It is possible that a combination of transient elastography and one of the aforementioned scoring systems may provide better performance than each of them used alone, although this needs to be verified in future studies.

This study had several limitations. First, the proportion of subjects with advanced fibrosis was small. Second, the patients were recruited from hepatology centers in Japan with a particular interest in the study of NAFLD; therefore, the possibility of some referral bias cannot be ruled out. Patient selection bias could also have existed, because liver biopsy might have been considered for NAFLD patients who were likely to have NASH. The findings may thus not represent those of the NAFLD patients in the community at large. The question remains as to whether the revised cutoff values of the various scoring systems might be useful in real clinical practice. Another limitation is that the supposedly normal range of ALT values is incorrect. The public health implications and clinical usefulness of reducing the upper limits of the normal value for the serum ALT continue to be under debate, and the currently proposed cutoff values for the upper limits of the serum ALT levels are 30 IU/l for men and 19 IU/l for women [39]. Recently, the upper limit of the normal range of serum ALT levels in the Asian population was reported as 35 IU/l for men and 26 IU/l for patients with a normal liver

histology [40]. According to our preliminary data, the AUROC calculated for detecting advanced fibrosis was 0.907 (FIB4 index), 0.916 (NAFLD fibrosis score), 0.793 (BARD), and 0.859 (AAR) in 127 biopsy-proven NAFLD patients with ALT ≤30 (data not shown). We also acknowledge that the pathologic diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling errors and/or inter-observer variability [41, 42].

In conclusion, the issue of development of a non-invasive method for the assessment of disease severity remains crucial in patients with NAFLD given the high number of subjects with steatosis and normal serum ALT values in the general population. We reset the cutoff values of numerous non-invasively determined indices to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal serum ALT values. In the absence of biopsy or of an adequate score capable of identifying subjects at risk, these patients could miss being included in the list for careful follow-up and might be scarcely motivated to adopt lifestyle modifications that could potentially cure their liver disease. Clinicians should be aware of the importance of complete clinical evaluation for early diagnosis and treatment of liver diseases. Non-invasive scoring systems, especially the FIB-4 index and the NAFLD fibrosis score showed high sensitivity and specificity, and they can be reliably used to exclude advanced fibrosis in NAFLD subjects with normal serum ALT levels.

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Original Paper

Long-Term (≥2 Yr) Efficacy of Vitamin E for Non-Alcoholic Steatohepatitis

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ABSTRACT

Background/Aims: Vitamin E is one of the most promising treatments for non-alcoholic steatohepatitis (NASH). However, the long-term efficacy of this treatment remains unknown. **Methodology:** We retrospectively examined 17 patients with biopsy-proven NASH who received vitamin E at a dose of 300 mg/day for ≥2 yr, and underwent second liver biopsies after treatment. Variables were compared between patients with (group R) and without (group NR) fibrosis regression. **Results:** The median interval between basal and second liver biopsies was 2.4 yr (range, 2.0–5.8 yr). Overall, transaminase activities, insulin resistance index, and hepatic fibrosis markers were significantly improved. Although histological steatosis, inflammation, and fibrosis did not change after treatment, liver fibrosis improved in seven patients (41.2%), progressed in five (29.4%), and remained unchanged in five (29.4%). At baseline, subjects in group R (n = 7) were more likely to have diabetes, insulin resistance, and severe fibrosis compared to those in group NR (n = 10). Lower NAFLD activity score and larger decrease of ALT and insulin resistance after treatment were observed in group R compared with group NR. **Conclusions:** Two years or longer treatment can be expected to ameliorate NASH fibrosis, especially in those whose serum transaminase activities and insulin resistance can be improved.

Key Words: Insulin resistance; Liver biopsy; Liver fibrosis; Oxidative stress.

Abbreviations: Non-Alcoholic Steatohepatitis (NASH); Non-Alcoholic Fatty Liver Disease (NAFLD); Aspartate Aminotransferase (AST); Alanine Aminotransferase (ALT).

INTRODUCTION

Non-alcoholic 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 non-alcoholic steatohepatitis (NASH), which can progress to liver cirrhosis (LC) and hepatocellular carcinoma (HCC) in the absence of significant alcohol consumption (1-4). Oxidative stress is postulated to play a central role in the pathogenesis of NASH (5). We have previously reported that levels of serum thioredoxin (TRX), an indicator of oxidative stress, are elevated in NASH patients compared to those with simple steatosis or healthy controls (6). Lipid peroxidation stimulates cytokine production, which leads to inflammation and activation of hepatic stellate cells, which enhance fibrosis. There is currently no treatment that is of proven benefit for NASH. Preliminary studies have provided a rationale for the use of lifestyle intervention, bariatric surgery, phlebotomy, and a variety of drugs. However, the usefulness of each of these therapeutic options has not been validated in rigorously performed, randomized controlled trials. Recently, the Non-alcoholic Steatohepatitis Clinical Research Network (NASH CRN) has conducted the Pioglitazone versus Vitamin E versus Placebo for the Treatment of Non-diabetic Patients with Non-alcoholic Steatohepatitis (PIVENS) trial, a phase 3, multicenter, randomized, placebo-controlled, double-blind clinical trial of pioglitazone or vitamin E for the treatment of adults without diabetes who had biopsy-confirmed NASH. By histological analysis, vitamin E was superior to placebo with regard to the resolution of NASH, and there was no benefit of pioglitazone over placebo (7). There have been no studies that have demonstrated the long-term (≥2 yr) efficacy of vitamin E for the treatment of NASH. Thus, the aim of this study was to evaluate clinical and histological parameters after ≥2 yr vitamin E treatment for NASH.

METHODOLOGY

Study population

A total of 135 patients were diagnosed with NASH during 2002–2008 at the Center for Digestive and Liver Diseases, Nara City Hospital. Sixty-nine of these received treatment with vitamin E. Fifty-one patients continued treatment and 18 stopped; four were discontinued by physicians and 14 were lost to follow-up. Seventeen patients refused or hesitated to undergo post-treatment biopsies, and 17 patients had treatment duration <2 yr. Finally, 17 patients who underwent liver biopsies after ≥2 yr treatment were involved in the present study (**Figure 1**, **Table 1**). All patients were given vitamin E (α-tocopherol; Eisai Co. Ltd., Tokyo, Japan) at 300 mg/day orally. The diagnosis of NAFLD was based on the following criteria: i) liver biopsy showing steatosis in at least 5% of hepatocytes (8); and ii) appropriate exclusion of liver diseases of other etiology, 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 who consumed >20 g/day alcohol and those with evidence of decompensated LC or HCC were excluded. Written informed consent was obtained from all patients at the time of their liver biopsy, and the study was conducted in accordance with the Helsinki Declaration. Informed consent was obtained from all patients. All

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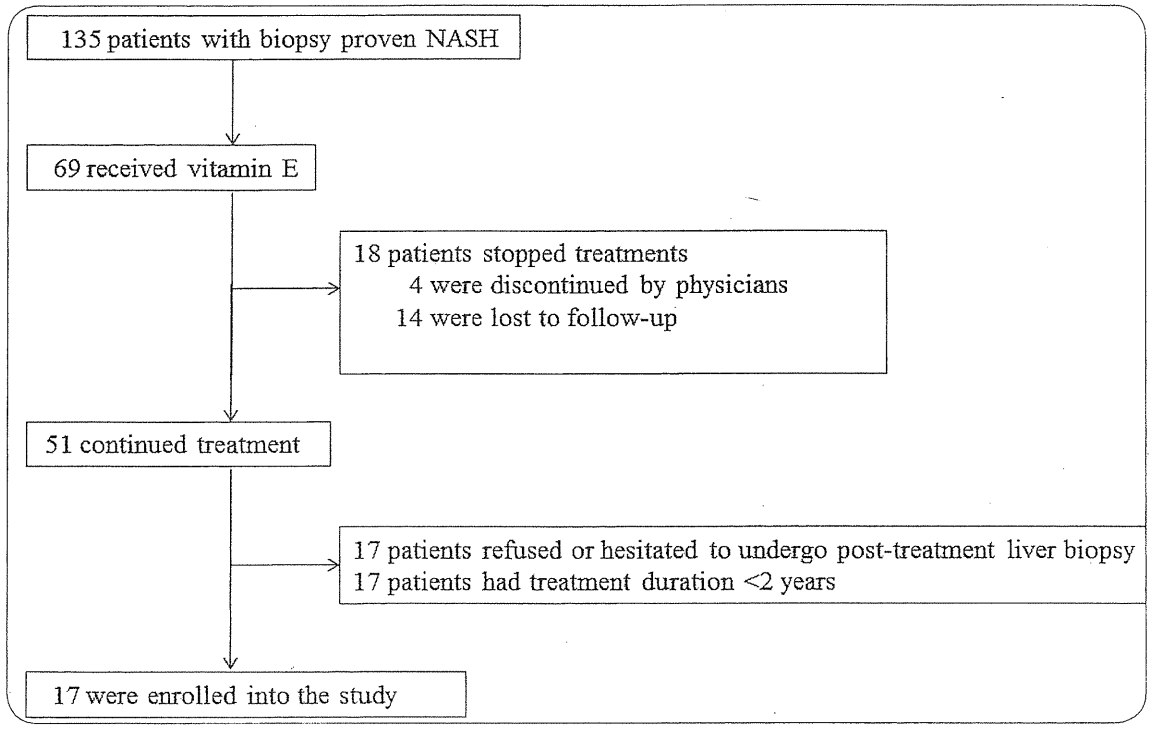

FIGURE 1. Patient selection.

subjects were given a standardized set of pragmatic recommendations about lifestyle changes and diet.

Anthropometric and laboratory evaluation

Venous blood samples were taken in the morning after a 12-h overnight fast. Laboratory evaluation in all patients included a blood cell count and measurement of aspartate aminotransferase (AST), alanine aminotransferase (ALT), fasting plasma glucose (FPG), immunoreactive insulin (IRI), and type IV collagen 7S. These were measured using the standard clinical chemistry techniques. Body mass index (BMI) was also calculated. Obesity was defined as BMI >25 kg/m2, according to the criteria of the Japan Society for the Study of Obesity (9). Patients were assigned a diagnosis of diabetes mellitus (DM) if they had documented use of oral hypoglycemic medication, a random glucose level >200 mg/dL, or FPG >126 mg/dL (10). The homeostasis model assessment for insulin resistance (HOMA-IR) was calculated on the basis of FPG and insulin according to the HOMA model formula: HOMA-IR= IRI (μU/mL) × FPG (mg/dL)/405 (11). The HOMA-IR model has been validated and widely used for determining the degree of insulin resistance, and strongly predicts the development of type 2 diabetes. Genomic DNA was extracted from whole blood using a DNA purification kit (FlexiGene DNA kit; QIAGEN, Hilden, Germany). The haptoglobin (HP) polymorphism was determined using PCR as previously described (12). Achievement of a reduction in ALT to either <35 IU/L or <50% from baseline was defined as improvement.

Histological evaluation

All patients enrolled in this study underwent a percutaneous liver biopsy under ultrasonic guidance. The liver specimens were embedded in paraffin and stained with hematoxylin and eosin, Masson–trichrome, and reticulin silver stain. Two hepatopathologists (S.I. and Y.S.) who were blinded to the clinical data reviewed the liver biopsy specimens. If our diagnosis is discordant, final diagnoses were determined through discussions between us. Fatty liver was defined as the presence of >5% steatosis, and steatohepatitis was diagnosed by steatosis, inflammation, and hepatocyte ballooning (2,3,8). NASH was defined as steatosis with lobular inflammation and ballooning degeneration, with or without Mallory–Denk bodies or fibrosis (2,3,8). The presence or absence of hepatocyte ballooning degeneration was influenced by the variability in the pathologists' interpretation. The NAFLD Activity Score (NAS) proposed by Kleiner *et al.* was the unweighted sum of the scores for steatosis (0–3), lobular inflammation (0–3), and ballooning degeneration (0–2) (8). The specific inclusion criteria were definite or possible steatohepatitis with an activity score of ≥5, or definite steatohepatitis (confirmed by two pathologists) with an activity score of three or four. The biopsied tissues were scored for steatosis, stage, and grade, according to the standard criteria for grading and staging of NASH proposed by Brunt *et al.* (13). Regression of liver fibrosis was defined as an improvement by one grade or more in the final stage with respect to the basal biopsy.

Statistical analyses

Continuous variables were expressed as median (range). Qualitative data were presented as numbers with percentages in parentheses. Statistical differences in quantitative data were determined using the Mann-Whitney *U* test. Fisher's exact probability test or $\chi 2$ analysis was used for qualitative data (**Tables 2** and **3**). Patients were separated in two groups. Group R patients were shown to have fibrosis regression in the second biopsy with respect to the basal biopsy; group NR patients were without fibrosis regression. All the variables were compared between the two groups using a Wilcoxon rank-sum test for the numerical variables, and a

TABLE 1. Patient characteristics at baseline and laboratory and histological changes after vitamin E treatment.

Case	Gender/age (years)	DM	HP	BMI (kg/m²)	ALT (IU/L)	HOMA-IR	IV-7S (ng/mL)	Steatosis	NAS	Brunt grade	Brunt stage
1	F/58	yes	2,1	33.8→32.0	218→39	6.96→3.36	6.4→4.9	1→2	5→4	2→1	3→2
2	F/74	yes	2,1	32.8→31.9	56→23	6.39→3.71	3.9→3.3	2→1	6→3	2→1	2→1
3	F/56	yes	2,2	26.3→25.9	57→27	12.47→4.32	5.6→3.9	2→2	4→4	1→1	3→2
4	F/66	yes	2,2	27.4→27.4	76→31	7.26→5.87	6.9→4.6	1→2	3→4	1→1	3→2
5	F/75	yes	2,2	19.6→19.3	69→23	4.36→3.07	8.9→2.6	1→1	5→3	2→1	3→2
6	F/56	yes	2,2	38.6→37.8	115→46	33.04→5.75	6.7→4.8	1→1	3→3	1→1	2→1
7	M/24	yes	ND	30.4→25.2	225→32	6.54→1.73	6→3.9	2→1	4→3	1→1	2→1
8	F/64	no	2,1	27.6→28.0	116→51	6.02→2.79	7.3→5.7	2→2	6→6	2→2	3→3
9	F/67	yes	ND	21.1→21.1	56→32	ND→2.19	5.3→3.6	1→1	5→5	2→2	2→2
10	F/75	yes	2,1	25.3→26.7	17→9	3.67→11.6	5.1→4.1	3→2	7→4	2→1	2→2
11	M/77	no	2,2	18.9→20.0	73→49	0.35→0.15	4.3→2.9	1→0	3→0	1→0	1→1
12	F/67	no	ND	24.7→25.3	146→251	4.49→4.07	6.3→2.8	1→1	5→3	2→1	1→1
13	M/71	no	2,1	24.1→23.7	74→14	4.71→3.63	7.4→4.3	1→1	3→3	1→1	2→3
14	M/55	yes	2,1	26.3→26.7	78→119	1.81→1.34	5.6→6.2	1→2	3→4	1→1	2→3
15	F/68	yes	2,2	29.7→28.6	57→56	7.84→3.00	5.3→3.5	1→3	5→5	2→1	1→2
16	F/80	no	2,2	32.8→30.5	65→35	4.96→3.30	5.9→5.2	2→1	4→5	1→2	2→3
17	F/68	yes	2,2	24.2→24.7	160→61	5.73→3.27	5.8→3.9	1→2	3→6	1→2	1→2

IV-7S: type IV collagen 7S, ND: not determined.

difference of two binomial proportions for the categorical variables. Numerical variables were compared at the time of basal liver biopsy and the second liver biopsy. Furthermore, gradient values (second *vs.* basal) were also compared between the two groups. Differences were considered statistically significant at $p < 0.05$.

RESULTS

Clinical characteristics of 17 patients enrolled for this study are shown in **Table 1**. Median age was 67 yr (range, 24–80 yr). Thirteen (76.5%) were women, 12 (70.6%) had DM, and 11 (64.7%) were obese (BMI >25 kg/m2). HP polymorphisms were determined in 14 patients; six patients had HP2/HP1, and 8 had HP2/HP2.

BMI did not significantly change after vitamin E treatment [26.3 (18.9–38.6) at baseline *vs.* 26.7 (19.4–37.8) after treatment, $P = 0.3066$). Body weight reduction was found in 9 of 17 patients, and body weight gain in 6. Compared with the pretreatment values, serum ALT and HOMA-IR decreased significantly post-treatment from 74 (17–225) to 35 (9–251) IU/l ($p = 0.0113$) and from 5.9 (0.4–33.0) to 3.3 (0.2–11.6) ($p = 0.0052$), respectively. ALT improvement was found in 10 of 17 patients. Type IV collagen 7S levels post-treatment [3.9 (2.6–6.2) ng/mL] were significantly lower than those at baseline [5.9 (3.9–8.9) ng/mL, $p = 0.004$).

Overall, histological steatosis, NAS, histological grade and fibrosis did not change significantly after vitamin E treatment. Liver fibrosis improved in seven patients (41.2%), progressed in five (29.4%), and remained unchanged in five (29.4%). Thus, we compared clinical characteristics and laboratory data between patients with (group R, n = 7) and without (group NR, n = 10) fibrosis regression (**Tables 2** and **3**). There were no significant differences between groups R and NR with regard to gender, age, and HP polymorphisms. There were no significant differences in the span between basal and second liver biopsy between group R (median, 2.3 yr; range, 2.0–2.8 yr) and NR (median, 2.6 yr; range, 2.0–5.8 yr). DM was more prevalent in group R than group NR (100% *vs.* 50%, $p = 0.0441$). ALT improvement was found in all patients in group R but only in four (40%) of 10 patients in group NR. Reductions in HOMA-IR and type IV collagen 7S were observed in all patients in group R. Histological features were compared before and after treatment with vitamin E between groups R and NR. At baseline, NAS, steatosis, and histological grade were similar between the two groups. Fibrosis stage was more severe in group R than in group NR. After treatment, only NAS was significantly lower in group R (median, 3.0; range, 3.0–4.0) than in group NR (median, 4.5; range 0.0–6.0, $p = 0.0147$).

TABLE 2. Baseline and final clinical features and gradients of laboratory markers associated with changes in liver fibrosis in 17 patients with NASH.

Variables	Regression(n=7)	No regression (n=10)	*p*
Gender (female)	6 (86%)	7 (70%)	0.6029
Age (years)	60 (24, 75)	68 (56, 80)	0.1067
HP 2-1/2-2/ND	2 (29%)/4 (57%)/1 (14%)	4 (40%)/4 (40%)/2 (20%)	0.6270
DM (yes)	7 (100%)	5 (50%)	0.0441
Interval (years)	2.3 (2.0, 2.8)	2.6 (2.0, 5.8)	0.2200
Baseline			
BMI (kg/m^2)	30.4 (19.6, 38.6)	27.4 (19.4, 37.8)	0.0875
ALT (IU/l)	76 (56, 225)	74 (17, 160)	0.5577
HOMA-IR	7.0 (4.4, 33.0)	4.7 (0.4, 7.8)	0.0229
Type IV collagen 7S (ng/mL)	6.4 (3.9, 8.9)	5.7 (4.3, 7.4)	0.3049
Final			
BMI (kg/m^2)	27.4 (19.6, 37.8)	26.0 (19.9, 30.5)	0.2831
ALT (IU/L)	31 (23, 46)	50 (9, 251)	0.1069
HOMA-IR	3.7 (1.7, 5.9)	3.1 (0.2, 11.6)	0.1719
Type IV collagen 7S (ng/mL)	3.9 (2.6, 4.9)	4.0 (2.8, 6.2)	0.7691
ΔBW (kg)	−2.1 (−17.2, 0)	−1.1 (−7.1, 5.4)	0.4321
ΔALT% (%)	−60 (−86, −53)	−45 (−81, 72)	0.0147
ΔHOMA-IR	−3.6 (−27.3, −1.3)	−1.1 (−4.8, 7.9)	0.0390
Δtype IV collagen 7S (ng/mL)	−1.9 (−6.3, −0.6)	−1.7 (−3.5, 0.6)	0.3404

ND: not determined. Values are median (min, max), counts (%), as appropriate. ALT % was calculated by the formula; (post-treatment ALT; pre-treatment ALT)/ pre-treatment ALT × 100 (%). p values were calculated by Mann-Whitney, or $\chi 2$ analysis, as appropriate.

Histological grades after treatment were grade 1 in all patients in group R, whereas four grade 2, five grade 1, and one grade 0 in group NR. A multivariate analysis was not performed because of the small sample size of the groups. During the follow-up period, no patients developed HCC or liver-related deaths.

DISCUSSION

This retrospective analysis revealed that the long-term (≥2 yr) treatment with oral vitamin E ameliorated hepatic liver enzymes, insulin resistance and hepatic fibrosis marker in NASH patients. Moreover, this treatment can be expected to improve NASH fibrosis, especially in patients in whom serum transaminase activities and insulin resistance are decreased. The previously reported rates of progression of fibrosis in NAFLD patients without medication are very similar (14-18); around 40% of patients with NAFLD develop progressive fibrosis over 3–14 yr, around 40% remain stable, and <30% regress. A relatively higher number (41.2%) of patients in the present study regressed compared to those in previous studies. Age, insulin resistance, severe obesity, type 2 diabetes, high levels of AST, hypertriglyceridemia, and the presence of inflammation on the initial biopsy are risk factors for progressive fibrosis in NAFLD (14-18). Importantly, Adams et al. have revealed that changes in aminotransferase parallel those in steatosis and inflammatory features but not fibrosis stage, which indicates that it is important to reduce body weight to within the normal range (14). In Japan, Hamaguchi *et al.* showed that a change in glycemic control (ΔA1C), but not changes in HOMA-IR, was an independent predictor of the progression of liver fibrosis in NAFLD patients (19).

The NASH CRN PIVENS trial has shown that 43% of vitamin-E-treated patients (800 IU/day for 96 weeks) showed improvement in the histological features of NASH. On the other hand, vitamin E did not improve the proportion of subjects with improvement in fibrosis scores (7). In that study, however, fibrosis improvement was achieved in 41% of patients in the vitamin E group, which was similar to our results. It is important to note that not all subjects benefit from vitamin E. It remains unknown whether the benefits of vitamin E vary according to patient characteristics at enrollment or follow-up. In the PIVENS study, serum ALT levels at 96 weeks fell markedly in the vitamin E responders and less so in non-responders (20). In that study, histological responses to vitamin E in NASH patients did not appear to be due to the correction of an unrecognized vitamin E deficiency. Improvement in serum ALT levels was associated with improved histological response as defined by the pre-specified primary endpoint (20). Further studies are needed to establish characteristics of patients who are likely to respond to vitamin E and to identify mediators of this response.

It is also important to clarify clinical factors associated with fibrosis progression or regression, because fibrosis is the most important predictor of prognosis or carcinogenesis in NASH patients. Thus, we compared clinical characteristics between patients with (group R) and without (group NR) fibrosis regression. Lager decrease of ALT and HOMA-IR and lower NAS post-treatment suggest that relief of hepatic inflammation and insulin resistance is required to achieve improvement in histological fibrosis. In fact, none of six patients who

TABLE 3. Baseline and final clinical features and gradients of histological findings associated with changes in liver fibrosis in 17 patients with NASH.

Histological findings	Regression (n=7)	No regression (n=10)	*p*
Baseline			
NAS	4.0 (3, 6)	4.5 (3, 7)	0.8217
Steatosis (1/2/3)	4/3/0	7/2/1	0.7195
Brunt grade (1/2/3)	4/3/0	5/5/0	1.0000
Brunt stage (1/2/3)	0/3/4	4/5/1	0.0147
Final			
NAS	3.0 (3, 4)	4.5 (0, 6)	0.0147
Steatosis (0/1/2/3)	0/4/3/0	1/4/4/1	0.8251
Brunt grade (0/1/2/3)	0/7/0/0	1/5/4/0	0.1858
Brunt stage (1/2/3)	3/4/0	2/4/4	0.0827
ΔNAS	−1 (−3, 1)	0 (−3, 3)	0.4515
Δsteatosis	0 (−1, 1)	0 (−1, 2)	0.9178
ΔBrunt grade	0 (−1, 0)	0 (−1, 1)	0.5916

Values are median (min, max), counts, as appropriate. p values were calculated by Mann-Whitney, or $\chi 2$ analysis, as appropriate

gained body weight improved hepatic fibrosis. On the other hand, reduction of body weight was obtained in six of seven patients who showed improvement of hepatic fibrosis. This suggests that lifestyle modification is also essential, even during vitamin E treatment, to improve hepatic fibrosis. A small pilot study has suggested that combination of pioglitazone and vitamin E is superior to vitamin E alone for improving liver histology (21). In the present study, all patients in group R showed ALT improvement, and a decrease in HOMA-IR and type IV collagen 7s. In this way, reductions of ALT, insulin resistance, and fibrosis markers are essential to accomplish regression of hepatic fibrosis.

Vitamin E is known to have antioxidant effects and to suppress peroxidation (22), and it has been reported to inhibit hepatic transforming growth factor (TGF)-β1 gene expression (23), and to protect against liver fibrosis in an animal model (24). There has been a small number of studies in which hepatic fibrosis has been evaluated after vitamin E treatment. In Japan, a study with vitamin E administration (300 mg/day for 1 yr) has confirmed improvement in liver tests with reduction of plasma levels of TGF-β1. In that study, fibrosis was improved in 5 (55.6%) of 9 patients undergoing liver biopsies after vitamin E administration (25). A group of biopsy-proven NASH patients were randomized to vitamin E (1,000 IU/day) plus vitamin C (1,000 mg/day) or placebo for 6 months (26). The patients showed no improvement in serum aminotransferase levels but repeat liver biopsies at the end of the trial demonstrated decreased fibrosis within the vitamin-treated group (especially in patients with DM). This result is in agreement with our study, which demonstrated that DM was more prevalent in group R than in group NR. We hypothesize that vitamin E treatment can be expected to be more useful in NASH patients with DM compared to those without, but the mechanism of this effect remains unknown. The reason why significant regression of hepatic fibrosis in vitamin E group was not obtained over placebo in the PIVENS trial could be explained by the fact that non-diabetic NASH patients were included (7). In future, the benefits of vitamin E should be validated in NASH patients with DM.

In the present study, fibrosis stage on initial biopsy was a predictor of fibrosis rate, with higher stage being associated with a higher rate of regression. This may in part be explained by the fact that patients without fibrosis (stage 0) cannot regress, whereas patients with cirrhosis (stage 4) cannot progress in fibrosis stage. However, all of our patients had stage 1–3 fibrosis.

HP is an α2-glycoprotein acute phase reactant that binds to free hemoglobin and prevents iron loss and oxidative damage during intravascular hemolysis (27,28). Three major phenotypes, Hp1-1, Hp2-1, and Hp2-2 are the product of two closely related genes, HP1 and HP2. The HP 2-2 polymorphism is associated with increased production of oxygen free radicals. HP polymorphisms are known to be a determinant of efficacy of vitamin E. Treatment of patients with HP 2-2 polymorphism with vitamin E appears to be highly effective for reduction of cardiovascular events (29,30). In our study, however, the distribution of HP polymorphism did not differ between groups R and NR, although a larger number of patients should be evaluated for future analysis to draw firm conclusions.

Unexpectedly, most of the patients examined in our study showed a decrease in HOMA-IR. The mechanism for this remains unknown. In a previous rat model, it has been shown that a diet high in lipid hydroperoxide induced by vitamin E deficiency accelerates glucose intolerance through impairments of sensitivity and secretion of insulin (31). In a recent experimental study, vitamin E has been found to improve the free radical defense system potential and to prevent oxidative-stress-induced insulin resistance in rat L6 muscle cells (32). According to a pilot study by Yakaryyilmaz *et al.* (33), vitamin E treatment for 24 weeks achieved improvement of hepatic steatosis by reducing insulin resistance and peroxisome proliferator-activated receptor α expression in NASH patients. In contrast, no additional effects of vitamin E on insulin resistance have been observed in pediatric NAFLD (34,35). In the PIVES trial, insulin resistance was improved only in the pioglitazone group (7). Therefore, our decrease in HOMA-IR may not be explained by direct actions of vitamin E, but from lifestyle modifications.

Our study had a few limitations. First, we performed an uncontrolled trial, using a small number of patients in a single center. The low statistical power of this study should be considered when we evaluate the results. We acknowledge that pathological diagnosis was mainly determined using liver tissues derived from percutaneous liver biopsies, which are prone to sampling error or inter-observer variability (36,37). The median interval between basal and second liver biopsies was 2.4 yr. No patients developed HCC or liver-related mortality. In the future, we should examine whether vitamin E treatment prevents hepatic carcinogenesis or hepatic failure, and leads to improved overall survival rate in NAFLD patients (38).

In conclusion, vitamin E treatment for ≥2 yr can be expected to ameliorate hepatic fibrosis in NASH patients, if serum transaminase activities and insulin resistance are improved. During vitamin E treatment, serum ALT and HOMA-IR should be monitored to evaluate the efficacy of vitamin E for preventing hepatic fibrosis progression. Further combinations of vitamin E with insulin sensitizers to reduce oxidative stress and insulin resistance may prove more effective in slowing down disease progression to cirrhosis in NASH patients.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Type 2 diabetes mellitus is associated with the fibrosis severity in patients with nonalcoholic fatty liver disease in a large retrospective cohort of Japanese patients

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Abstract
Background The prevalence of nonalcoholic fatty liver disease (NAFLD) and metabolic syndrome have been increasing worldwide. The associations between metabolic factors and the histologic severity of NAFLD have not yet been clarified. Therefore, we studied the relationships between relevant metabolic factors and the histological severity of NAFLD.
Methods In a cross-sectional multicenter study conducted in Japan, we examined 1,365 biopsy-proven NAFLD patients. The frequencies of underlying lifestyle-related diseases and their relationships to the NAFLD histology were investigated.
Results The hepatic fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in the male patients, and 16.2/31.7/23.9/21.6/6.6 (%) in the female patients. Dyslipidemia was present in 65.7 % (hypertriglyceridemia, 45.3 %; increased low-density lipoprotein cholesterol, 37.5 %; decreased high density lipoprotein cholesterol, 19.5 %) of patients. Hypertension was present in 30.2 %, and diabetes mellitus (DM) in 47.3 %. The fibrosis stage increased with age, especially in postmenopausal females. The body mass index was positively correlated with the fibrosis stage. Deterioration of glucose control was positively correlated

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with the fibrosis stage, this correlation being more prominent in females. Multivariate analysis identified age and DM as significant risk factors for advanced fibrosis. No significant correlation of the fibrosis stage was observed with hypertension. There was a negative correlation between the serum triglyceride levels and the fibrosis stage. *Conclusions* DM appeared to be a significant risk factor for advanced fibrosis in patients with NAFLD, and would therefore need to be properly managed to prevent the progression of NAFLD.

Keywords NAFLD · Histology · Diabetes mellitus · Retrospective study

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
IR	Insulin resistance
DM	Diabetes mellitus
NAFL	Nonalcoholic fatty liver
BMI	Body mass index
CT	Computed tomography
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
ChE	Cholinesterase
HDL	High density lipoprotein
LDL	Low-density lipoprotein
FPG	Fasting plasma glucose
HbA1c	Hemoglobin A1c
FFA	Free fatty acid
CRP	C-reactive protein
IRI	Immunoreactive insulin
HOMA-IR	Homeostasis model assessment-insulin resistance
SD	Standard deviation
IGT	Impaired glucose tolerance
NGT	Normal glucose tolerance

Introduction

Nonalcoholic fatty liver disease (NAFLD) is one of the most commonly encountered chronic liver disease in the world. According to Japanese annual health check reports, 9–30 % of Japanese adults suffer from NAFLD [1–3]. Since it is now known that almost 10–20 % of individuals with NAFLD have nonalcoholic steatohepatitis (NASH), the prevalence of NASH is estimated to be 1–3 % in the adult Japanese population, similar to the prevalence reported from Western countries.

Nonalcoholic fatty liver disease includes a wide spectrum of liver diseases, ranging from nonalcoholic fatty liver (NAFL), a benign and non-progressive condition, to NASH, which can progress to liver cirrhosis and hepatocellular carcinoma even in the absence of a history of significant alcohol consumption [4–7]. Furthermore, NASH is considered to be the hepatic manifestation of metabolic syndrome, and has been shown to be associated with obesity, insulin resistance (IR) and abnormalities of glucose and lipid metabolism [8–16]. Importantly, the rates of nonalcoholic fatty liver (NAFL) and NASH are expected to continue to grow with the developing pandemic of obesity and diabetes mellitus, to become global public health concerns.

Owing to the difficulties in diagnosing NAFLD (NAFL and/or NASH) and referral bias, it has been difficult to determine the prognostic factors in patients with NAFLD. NAFLD is a complex disease with multiple etiopathogenetic factors, including obesity, type 2 DM, dyslipidemia, hypertension, and other diseases associated with metabolic dysregulations. Recent reports have suggested that DM is an independent risk factor for NAFLD [17–19]. Despite the high prevalence and potentially serious nature of this disease, relatively little is known about the metabolic factors that might be associated with the histological severity of NAFLD.

The purpose of this study was to conduct a retrospective investigation of the association between metabolic factors and the histologic severity of NAFLD in a large cohort of Japanese patients with NAFLD.

Patients and methods

Patient population

A total of 1,365 biopsy-proven NAFLD patients seen between 2001 and 2012 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following nine hepatology centers in Japan: Hiroshima University, Kyoto Prefectural University of Medicine, Yokohama City University, Kochi Medical School, Saga Medical School, Osaka City University, Nara City Hospital, Kurume University, and Saiseikai Suita Hospital. A portion of the patients (76.8 %; 1,048 out of 1,365) had also been involved in the previous JSG-NAFLD study [20, 21]. Informed consent was obtained from each patient, and the study was conducted in conformity with the ethical guidelines of the 7th revision of the Declaration of Helsinki (in October 2008) [22] and the approval of the ethics and research committees of the hospitals. In all patients, the current and past daily alcohol intake was less than 20 g per day; details regarding alcohol consumption were obtained independently by at least two physicians and confirmed by close family members. None

of the patients were receiving any medications that could cause NASH. Among the patients, those with the following disorders were excluded: secondary causes of steatohepatitis, drug-induced liver disease, alcoholic liver disease, viral hepatitis, autoimmune hepatitis, primary biliary cirrhosis, α1-antitrypsin deficiency, hemochromatosis, Wilson's disease, and biliary obstruction. [23].

Study design

A complete physical examination was performed on each patient within 1 month prior to the liver biopsy, as reported previously [24]. The body mass index (BMI) was calculated as the weight (kg) divided by height (m)-squared. Obesity was defined as a BMI of greater than 25, according to the criteria of the Japan Society for the Study of Obesity [25]. Computed tomography (CT) was used to determine the visceral fat area at the level of the umbilicus [26], as previously reported [24]. Dyslipidemia was diagnosed based on serum cholesterol levels higher than 220 mg/dl and/or high-density lipoprotein cholesterol levels lower than 40 mg/dl and/or triglyceride levels over 150 mg/dl. Hypertension was diagnosed if the patient was on antihypertensive medication and/or had a resting recumbent blood pressure of $\geq$ 130/85 mmHg on at least two occasions. Hyperuricemia was diagnosed based on serum uric acid levels higher than 7.0 mg/dl. DM was diagnosed according to the 2006 World Health Organization (WHO) criteria [27].

Venous blood samples were taken in the morning following overnight fasting for 12 h. The laboratory evaluation in all patients included a blood cell count, hemoglobin, platelet count; and the serum levels of aspartate aminotransferase (AST), alanine aminotransferase (ALT), AST/ALT ratio, lactate dehydrogenase (LDH), alkaline phosphatase (ALP), gamma glutamyl transpeptidase (GGT), cholinesterase (ChE), total bilirubin, direct bilirubin, albumin, total cholesterol, triglycerides, high density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, fasting plasma glucose (FPG), hemoglobin A1c (HbA1c), immunoreactive insulin (IRI), ferritin, uric acid, free fatty acid (FFA), and hyaluronic acids, were measured periodically during the treatment using the standard techniques of clinical chemistry laboratories.

Insulin resistance was calculated by the homeostasis model assessment-insulin resistance (HOMA-IR) using the following formula: HOMA-IR = fasting insulin (μU/ml) × plasma glucose (mg/dl)/405 [28].

Pathology

Patients enrolled in this study underwent percutaneous liver biopsy under ultrasonic guidance after obtaining informed consent. Formalin-fixed, paraffin-embedded liver sections were stained routinely with hematoxylin-eosin, silver reticulin, and Masson trichrome. All the specimens were examined by an experienced pathologist who was unaware of the clinical and biochemical data of the patients. Histological diagnosis for NAFLD was performed according to the methods of Matteoni et al. [6]. Grading and staging was classified according to Brunt et al. [29] and Kleiner et al. [30], as previously reported. In brief, steatosis was graded as follows: grade 1 (5–33 % of hepatocytes affected), grade 2 (34–66 % of hepatocytes affected), or grade 3 (> 66 % of hepatocytes affected). Necroinflammation was graded from grade 0 (absent) to 3 (1, occatonal ballooned hepatocytes and no or very mild inflammation; 2, ballooning of hepatocytes and mild-to-moderate portal inflammation; 3, intra-acinar inflammation and portal inflammation). Fibrosis was staged from grade 0 (absent) to 4 (1, perisinusoidal/pericellular fibrosis; 2, periportal fibrosis; 3, bridging fibrosis; 4, cirrhosis).

Statistical analyses

The data were statistically analyzed using R software, version 3.0.0. Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data are expressed as numbers, with percentages shown in parentheses.

Statistically significant differences in the quantitative data were determined using the *t* test or Mann–Whitney *U* test. Multivariate analysis was carried out by logistic regression. Differences were considered to be statistically significant at *P* values of less than 0.05.

Results

Patient characteristics

A total of 1,365 biopsy-proven patients with NAFLD were enrolled in this study. The demographic and clinical characteristics of the male and female NAFLD patients are shown in Supplemental Table 1. Of the total, 709 were males. The mean age of the patients was 51.0 ± 14.9 years (45.7 ± 15.1 and 56.8 ± 12.4 years for males and females, respectively). Whereas no significant differences were observed in the BMI, blood pressure, waist circumference, and visceral fat area between the male and female patients, the subcutaneous fat area and L/S ratio were significantly higher in the female patients. Statistically significant differences were observed in the white blood cell count, hemoglobin, and serum levels of transaminases, AST to ALT ratio, LDH, ALP, GGT, ChE, total and direct bilirubin, albumin, triglycerides, HDL cholesterol, fasting

Table 1 Prevalences of metabolic abnormalities in NAFLD patients

Variable	Percentage
BMI ≥ 25	73.0
Hypertension	39.9
Dyslipidemia	65.7
Hypertriglyceridemia	45.3
Hyper-LDL cholesterolemia	37.5
Hypo-HDL cholesterolemia	19.5
DM	47.3
Hyperuricemia	30.2

glucose, HbA1c, ferritin, uric acid, and hyaluronic acid between the male and female patients, as shown in Supplemental Table 1.

The frequencies of the metabolic abnormalities in the NAFLD patients are shown in Table 1. Obesity, as defined by the criteria of the Japan Society for the Study of Obesity, was seen in 73.0 % of the NAFLD patients, hypertension was found in 39.9 %, dyslipidemia in 65.7 % (hypertriglyceridemia, 45.3 %; hyper-LDL cholesterolemia, 37.5 %; hypo-HDL cholesterolemia, 19.5 %), type 2 diabetes in 47.3 %, and hyperuricemia in 30.2 % of the patients.

Distribution of the metabolic factors by the histological findings

The fibrosis stages (Stage 0/1/2/3/4) were 22.6/34.1/26.7/14.5/2.1 (%) in males, and 16.2/31.7/23.9/21.6/6.6 (%) in females, respectively. The distribution of the fibrosis stage in the different age groups in both genders is shown in Supplementary Fig. 1. Whereas the percentage of patients with advanced fibrosis (Stage 3 and 4) increased gradually with age in both genders, significant increase was seen after the age of 60 years in the females.

The prevalences of obesity (BMI ≥ 25) for each fibrosis stage are shown in Supplementary Fig. 2. The percentages of patients with obesity for each fibrosis stage (Stage 0/1/2/3/4) were 61.3/73.3/79.9/86.4/80.0 (%) in males, and 57.1/72.9/74.4/75.9/74.4 (%) in females, respectively. The prevalence of obesity showed a linear increase with progression of the fibrosis stage in the male NAFLD patients. However, no such increase was observed in the female NAFLD patients between Stage 1 and Stage 4.

The prevalences of dyslipidemia for each fibrosis stage are shown in Figs. 1 and 2. The percentages of patients with hypertriglyceridemia for each fibrosis stage (Stage 0/1/2/3/4) were 56.3/57.7/54.8/51.0/26.7 (%) in males, and 34.0/39.5/39.1/30.2/12.2 (%) in females, respectively. The percentages of patients with hyper-LDL cholesterolemia for each fibrosis stage (Stage 0/1/2/3/4) were 38.6/36.2/41.3/30.4/18.2 (%) in males, and 43.4/38.0/43.6/36.8/11.1 (%) in females, respectively. The prevalence rates of dyslipidemia (hypertriglyceridemia and hyper-LDL cholesterolemia) decreased with progression of the fibrosis stage, especially in Stage 4.

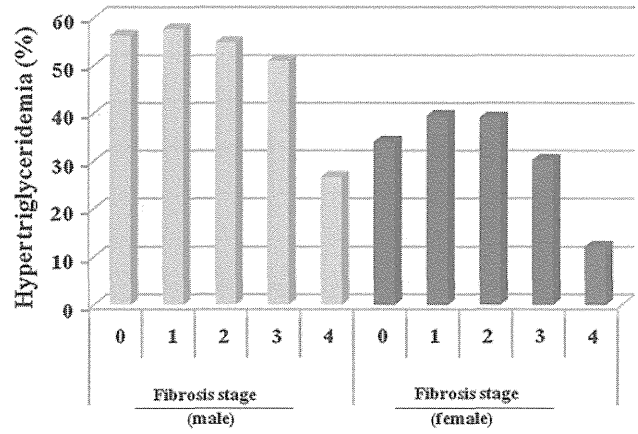

Fig. 1 Prevalence of hypertriglyceridemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertriglyceridemia

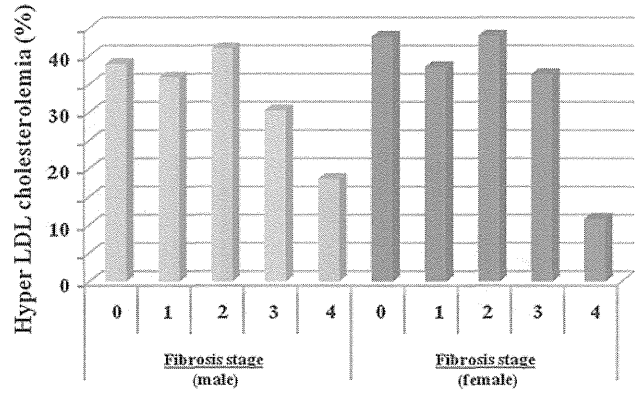

Fig. 2 Prevalence of hyper-LDL cholesterolemia for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hyper-LDL cholesterolemia

The prevalence of hypertension for each fibrosis stage was shown in Fig. 3. The percentages of patients with hypertension for each fibrosis stage (Stage 0/1/2/3/4) were 17.9/34.0/40.3/51.4/42.9/35.3 (%) in males, and 35.3/50.0/47.7/50.0/23.9 (%) in females respectively.

The prevalences of impaired glucose tolerance, including DM, for each fibrosis stage are shown in Fig. 4. The percentages of patients with DM for each fibrosis stage (Stage 0/1/2/3/4) were 23.7/32.8/53.7/65.8 (%) in males, and 34.7/45.2/60.9/64.7 (%) in females, respectively. The percentages of patients with impaired glucose tolerance (IGT) in each fibrosis stage (Stage 0/1/2/3/4) were 6.6/18.5/17.6/16.2 (%) in males, and 15.3/10.6/14.1/14.1 (%) in females, respectively. The percentages of patients with

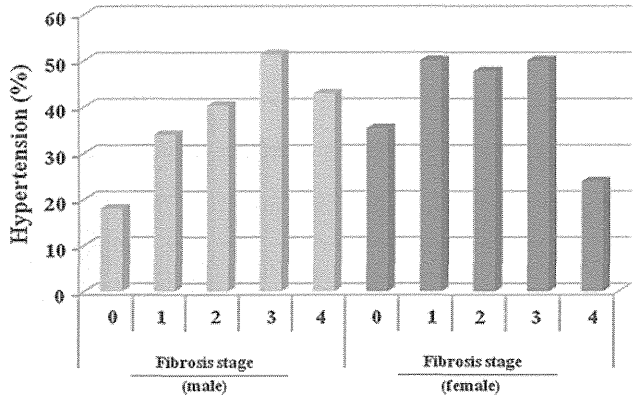

Fig. 3 Prevalence of hypertension for each stage of fibrosis. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the percentage of patients with hypertension

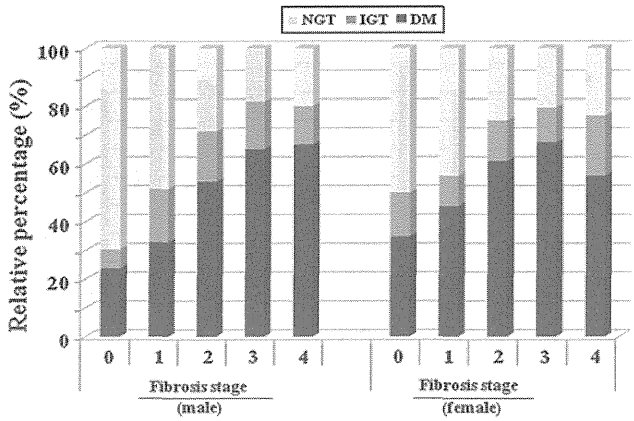

Fig. 4 The glucose tolerance pattern for each fibrosis stage in male and female NAFLD patients. The *horizontal axis* shows the fibrosis stage and the *longitudinal axis* shows the normal glucose tolerance, impaired glucose tolerance, or DM

normal glucose tolerance (NGT) were 69.7/48.7/28.7/17.9 (%) in males, and 50.0/44.2/25.0/21.2 (%) in females, respectively. The percentage of patients with DM increased with progression of the fibrosis stage in both male and female NAFLD patients.

Factors associated with advanced fibrosis

Factors associated with advanced fibrosis were examined (Table 2). NAFLD patients with advanced fibrosis were older, more likely to be female, and obese. The BMI, visceral fat area, and liver/spleen (L/S) ratio were significantly higher in NAFLD patients with advanced fibrosis. Furthermore, significant increases of the serum level of AST, AST/ALT ratio, ALP, GGT, total and direct bilirubin, fasting glucose, HbA1c, IRI, HOMA-IR, ferritin, FFA, and hyaluronic acid, and decreases of hemoglobin, platelet count, ChE, albumin, total cholesterol, triglycerides, LDL cholesterol, and uric acid were observed in the patients with advanced fibrosis. In cases with high fasting plasma glucose levels, HOMA-IR does not reflect insulin resistance exactly, and was assumed to be a reference level. To investigate the factors that might be related to the progression to advanced fibrosis, univariate analysis was performed between NAFLD patients with advanced fibrosis and those with no or mild fibrosis, as shown in Table 3. The results of the analysis revealed obesity (BMI $\geq$ 25), hypertension, hypotriglyceridemia, hyper-LDL cholesterolemia, DM, and hyperuricemia as risk factors for advanced fibrosis. Multivariate analysis identified older age, low serum triglyceride and DM as risk factors for advanced fibrosis.

Discussion

Many factors have been reported to be implicated in the pathogenesis of NAFLD, including obesity, DM, dyslipidemia and hypertension. However, it is still unclear how the metabolic factors might affect the pathogenesis and progression of NAFLD [11, 20, 31–34]. Therefore, identifying the risk factors for the deterioration of NAFLD would be useful for designing therapeutic strategies not only for the liver itself, but also for these metabolic diseases. Whereas a large number of papers have reported the differences in the clinical features between NAFL and NASH, comparisons of the clinical features by the histological severity are scarce. In this study, we retrospectively investigated the associations between metabolic factors and the histologic severity of NAFLD in a large cohort of 1,365 biopsy-proven NAFLD patients, considered as one of the largest-scale studies in the world to date.

The first important finding of our study was that the severity of fibrosis advanced gradually with age in the male patients with NAFLD, while it increased only in those women over 60 years of age. This gender difference may be attributable to menopause in females [35, 36].

The second important finding of our study was the association between obesity and fibrosis severity in NAFLD patients. We compared the prevalence of obesity and the histological severity of NAFLD. As shown in Supplementary Fig. 2, whereas the prevalence of obesity increased with the progression of fibrosis in males, the prevalence remained at approximately 70 % in all age groups of females.

It has been reported that 42–72 % of patients with NAFLD, including NASH, have dyslipidemia [37, 38]. Consist with these reports, dyslipidemia was present in 65.7 % of patients in our study, including hypertriglyceridemia in 45.3 %, increased serum low-density lipoprotein cholesterol in 37.5 %, and decreased serum high-density

Table 2 Comparison for the demographic and clinical characteristics between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis with NAFLD

Variable	All cases (n = 1,365)	Stage 0–2 (n = 1,062)	Stage 3, 4 (n = 303)	P value	P value (after adjustment for age/sex)
Age	51 ± 14.9	49 ± 15.0	57 ± 12.8	<0.0001	
Gender (male/female)	709/656	591/471	118/185	<0.0001	
Clinical and anthropometric measure					
Body mass index (kg/m^2)	27.9 ± 4.8	27.7 ± 4.8	28.6 ± 4.7	0.0006	<0.0001
BMI ≥ 25 (%)	73.0	71.2	79.5	0.0054	<0.0001
Waist circumference (cm^2)	96.7 ± 13.5	96.1 ± 12.4	98.1 ± 15.4	0.2372	0.0239
Subcutaneous fat area (cm^2)	220.7 ± 103.9	221.1 ± 110.7	219.7 ± 87.1	0.4631	0.4865
Visceral fat area (cm^2)	151.9 ± 65.9	144.4 ± 56.8	168.5 ± 80.3	0.0025	0.0007
L/S ratio	0.75 ± 0.30	0.73 ± 0.29	0.81 ± 0.32	0.0013	0.3528
Blood pressure sys. (mmHg)	127 ± 16.9	127 ± 15.7	124 ± 21.3	0.5343	0.0867
Blood pressure dia. (mmHg)	77 ± 11.1	77 ± 11.0	76 ± 11.5	0.6701	0.6802
Laboratory studies					
White blood cells (/μl)	6,330 ± 1,616.9	6,348 ± 1,583.4	6,272 ± 1,717.7	0.7037	0.6377
Hemoglobin (g/dl)	14.5 ± 1.6	14.6 ± 1.6	14.2 ± 1.6	<0.0001	0.6617
Platelet count (×10^4/μl)	22.4 ± 10.0	23.7 ± 10.4	18.0 ± 6.7	<0.0001	<0.0001
AST (IU/l)	57 ± 38.9	52 ± 36.0	72 ± 44.6	<0.0001	<0.0001
ALT (IU/l)	88 ± 60.3	87 ± 60.2	92 ± 60.4	0.1319	0.0003
AST/ALT	0.72 ± 0.3	0.67 ± 0.3	0.89 ± 0.4	<0.0001	<0.0001
LDH (IU/l)	210 ± 55.1	209 ± 56.7	213 ± 49.9	0.1446	0.5835
ALP (IU/l)	258 ± 111.0	250 ± 103.5	284 ± 130.1	<0.0001	0.0003
GGT (IU/l)	91 ± 103.4	88 ± 103.2	101 ± 103.4	<0.0001	0.0023
ChE (IU/l)	374 ± 106.5	383 ± 104.7	345 ± 107.3	<0.0001	0.0004
Bilirubin, total (mg/dl)	0.89 ± 0.39	0.86 ± 0.36	0.97 ± 0.45	0.0024	0.7731
Bilirubin, direct (mg/dl)	0.21 ± 0.16	0.19 ± 0.13	0.26 ± 0.22	<0.0001	0.2974
Albumin (g/dl)	4.46 ± 0.43	4.50 ± 0.39	4.29 ± 0.50	<0.0001	<0.0001
Total cholesterol (mg/dl)	209 ± 41.9	212 ± 41.5	200 ± 41.6	<0.0001	<0.0001
Triglyceride (mg/dl)	164 ± 102.6	170 ± 107.7	145 ± 79.7	<0.0001	0.0226
HDL cholesterol (mg/dl)	51 ± 15.7	51 ± 16.2	51 ± 13.9	0.3545	0.0297
LDL cholesterol (mg/dl)	130 ± 37.9	133 ± 37.2	123 ± 38.8	<0.0001	0.0009
Fasting plasma glucose (mg/dl)	114 ± 37.8	111 ± 36.1	123 ± 41.4	<0.0001	0.0001
HbA1c (NGSP) (%)	6.32 ± 1.2	6.26 ± 1.2	6.67 ± 1.4	<0.0001	0.0003
IRI (μIU/ml)	15.2 ± 18.5	13.7 ± 11.7	20.1 ± 31.4	<0.0001	<0.0001
HOMA-IR	4.89 ± 10.0	3.98 ± 5.6	6.84 ± 15.5	<0.0001	0.0012
Ferritin (ng/ml)	260.2 ± 475.8	255.8 ± 522.4	275.4 ± 254.5	0.5642	0.5421
Uric acid (mg/dl)	5.9 ± 1.5	6.0 ± 1.5	5.7 ± 1.3	0.0297	0.8563
Free fatty acid (μEq/l)	0.41 ± 0.3	0.36 ± 0.3	0.56 ± 0.3	<0.0001	<0.0001
Hyaluronic acid (ng/ml)	64.3 ± 168.9	42.2 ± 66.9	145.8 ± 329.9	<0.0001	<0.0001

Comparison between patients with mild (Stage 0–2) and advanced (Stage 3, 4) fibrosis using the Chi-square test for binary variables and logistic regression of group indicator on continuous variables

lipoprotein cholesterol in 19.5 % of patients. However, as the third important finding of our study, dyslipidemia tended to decrease in prevalence as the fibrosis stage progressed. Multivariate analysis revealed a negative correlation between the serum triglyceride levels and the fibrosis stage (OR = 0.5687, 95 % CI 0.394–0.821). This result may reflect a deterioration of lipid metabolism with the progression of liver fibrosis towards liver cirrhosis.

The fourth finding of our study was the recognition of a relationship between hypertension and the fibrosis severity in NAFLD patients. In our NAFLD population, hypertension was present in 30.2 %. Whereas no obvious trends in

Table 3 Multiple regression analysis to identify predictive factors for the advanced fibrosis

Variable	All cases	Stage 0–2	Stage 3, 4	Univariate odds ratio (95 % CI)	*P* value	Multivariate odds ratio (95 % CI)	*P* value
Age (mean)	51.0	49.2	57.5	1.042 (1.032–1.053)	<0.0001	1.036 (1.021–1.051)	<0.0001
Female (%)	48.1	44.4	61.1	1.967 (1.516–2.553)	<0.0001	1.180 (0.787–1.768)	0.423
BMI ≥ 25 (%)	73.0	71.2	79.5	1.566 (1.149–2.133)	0.0045	1.568 (0.991–2.481)	0.0545
Hypertension (%)	39.9	38.0	47.3	1.468 (1.063–2.027)	0.0198	0.943 (0.641–1.387)	0.7640
Hypertriglyceridemia (%)	45.3	82.7	34.7	0.566 (0.432–0.739)	<0.0001	0.663 (0.453–0.970)	0.0343
Hyper-LDL cholesterolemia (%)	37.5	39.6	30.7	0.676 (0.496–0.920)	0.0129	0.885 (0.596–1.313)	0.5444
Hypo-HDL cholesterolemia (%)	19.5	19.7	18.9	0.836 (0.671–1.343)	0.7680		–
DM (%)	47.3	42.1	64.9	2.544 (1.948–3.320)	<0.0001	2.387 (1.603–3.553)	<0.0001
Hyperuricemia (%)	30.2	32.1	24.4	0.684 (0.485–0.965)	0.0308	1.058 (0.693–1.617)	0.793

the prevalence of hypertension were observed in females, comparison of the relationship between the prevalence of hypertension and the stage of fibrosis, except for Stage 4, revealed a tendency towards increase in the prevalence of hypertension with progression of the fibrosis stage. In general, blood pressure is considered to have an effect on the rate of progression of NAFLD. Systolic and diastolic blood pressures have been reported to be correlated with the liver fat content, and patients with systolic hypertension were reported to be correlated with the liver fat contents, and patients with systolic hypertension were reported to show a two-fold higher risk of development of NAFLD [39]. As shown in Fig. 3, the decrease in the rate of hypertension in NAFLD patients with Stage 4 liver fibrosis might be, at least in part, attributable to the hyperdynamic circulation, characterized by peripheral vasodilation and increased portal resistance, observed in patients with liver cirrhosis [40, 41].

Impaired glucose tolerance is well known to accompany NAFLD. While it appears clear that abnormal glucose tolerance, including DM, is a risk factor for NAFLD and vice versa, the relationship between abnormal glucose tolerance and the histological severity of NAFLD is still unknown. The fifth finding in our study was that the prevalence of DM increased with progression of the fibrosis stage (Fig. 4). Multivariate analysis identified DM as an independent risk factor for advanced fibrosis (OR = 2.8573, 95 % CI 1.941–4.207). In vitro, high glucose and high insulin concentrations, which are often observed in patients with NAFLD, were shown to stimulate connective tissue growth factor expression, which is known as one of the important mechanisms involved in the progression of hepatic fibrosis [42]. Furthermore, the cirrhotic condition is suspected to facilitate the development of hyperinsulinemia and hyperglycemia via the deteriorated liver function [43, 44]. Taken together, it would be reasonable to consider DM as both a cause and result of NAFLD [45].

In conclusion, we have reported the prevalences of lifestyle-related diseases, such as obesity, dyslipidemia, hypertension, and DM, in NAFLD patients according to the stage of fibrosis. Multivariate analysis identified DM as a significant risk factor for advanced fibrosis. Accordingly, impaired glucose tolerance, including DM, should be properly evaluated and managed for preventing the progression of NAFLD, even in the early stages of NASH.

Conflict of interest The authors declare that they have no conflict of interest.

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