Conclusions We reset the cutoff values of numerous non-invasive scoring systems to improve their clinical usefulness in the prediction of liver fibrosis in NAFLD patients with normal ALT, and these non-invasive scoring systems with the reset cutoff values could be of substantial benefit to reduce the number of liver biopsies performed.

Keywords NAFLD · NASH · Normal ALT · Scoring systems

Abbreviations

NAFLD	Nonalcoholic fatty liver disease
NASH	Nonalcoholic steatohepatitis
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
AUROC	Area under the receiver-operating characteristic curve
BMI	Body mass index
LDL	Low-density lipoprotein
HDL	High-density lipoprotein
NPV	Negative predictive value
PPV	Positive predictive value
AAR	AST/ALT ratio

Introduction

Nonalcoholic fatty liver disease (NAFLD) is an important clinical subtype of chronic liver disease in many countries around the world [1]. The histological changes range over a wide spectrum, extending from simple steatosis, which is generally non-progressive, to nonalcoholic steatohepatitis (NASH), liver cirrhosis, liver failure, and sometimes even hepatocellular carcinoma [2–5]. The severity of liver fibrosis must be estimated to determine the prognosis, for surveillance, and for optimal treatment of NAFLD, similar to the case for other liver diseases [6]. Liver biopsy is recommended as the gold standard for the diagnosis and staging of fibrosis in patients with NASH [1, 2, 7]. This procedure, however, is invasive and is associated with a high risk of complications [8]. Approximately 24.6 % of all patients complain of pain during/after the biopsy procedure [9], and the estimated risk of severe complications is 3.1 per 1,000 procedures [10]. Furthermore, it is impossible to enforce liver biopsy in all NAFLD patients, because the estimated number of NAFLD patients has reached 80–100 million in the US and over 20 million in Japan [11]. These considerations underscore the need for the development of simple non-invasive methods for assessing the severity of fibrosis.

Numerous non-invasive panels of tests have been developed for the staging of liver disease consisting of combinations of clinical and routine laboratory parameters, as well as specialized tests, such as direct markers of fibrosis and elastography [12–15]. Especially serum alanine aminotransferase (ALT) has long been used as a surrogate marker of liver injury [16, 17] and has been used in many scoring systems for various liver diseases, including NAFLD, such as the aspartate aminotransferase (AST)-to-ALT ratio (AAR) [18], NAFLD fibrosis score [19], BARD score [20], and FIB-4 index [21]. It is, however, well known that both fatty liver and NASH may exist without elevation of the serum ALT value [22, 23]. It is also well known that the serum ALT values may not always be well correlated with the severity of liver disease [17].

The purpose of this study was to compare the distribution of histological fibrosis stage and scoring systems in various serum ALT levels and to investigate the clinical usefulness of established clinical scoring systems for detecting the presence of advanced liver fibrosis (bridging fibrosis or cirrhosis) and resetting the reported cutoff values, as appropriate, in a large retrospective cohort of Japanese patients with NAFLD patients with normal ALT levels.

Patients and methods

Patients

A total of 1,102 patients with liver-biopsy-confirmed NAFLD between 2002 and 2011 were enrolled from institutes affiliated with the Japan Study Group of NAFLD (JSG-NAFLD), represented by the following ten hepatology centers in Japan: Nara City Hospital, Yokohama City University, Hiroshima University, Kochi Medical School, Saga Medical School, Osaka City University, Kyoto Prefectural University of Medicine, Asahikawa Medical College, Kurume University, and Saiseikai Suita Hospital. We performed liver biopsy for the purpose of diagnosis and staging of NASH. The principal indications for liver biopsy were a persistent decrease of the platelet count and increase in the serum levels of the direct markers of fibrosis (type IV collagen 7s and hyaluronic acid) according to the consensus of the Japan Society of Hepatology (JSH). In addition, older age, presence of diabetes, obesity, a prolonged history of steatosis, and the results of elastography were also considered on an individualized basis. The histological criterion used for the diagnosis of NAFLD was the presence of macrovesicular fatty changes in the hepatocytes, with displacement of the nuclei to the edges of the cells [24]. The criteria for exclusion from this study included a history of hepatic disease, such as chronic hepatitis C or concurrent active hepatitis B (seropositive for hepatitis B

surface antigen), autoimmune hepatitis, primary biliary cirrhosis, sclerosing cholangitis, hemochromatosis, α1-antitrypsin deficiency, Wilson's disease, or hepatic injury caused by substance abuse, as well as a current or past history of consumption of more than 20 g of alcohol daily. Informed consent was obtained from each patient included in the study, and the study protocol conforms to the ethical guidelines of the 1975 Declaration of Helsinki as reflected in a priori approval by the institution's human research committee.

Anthropometric and laboratory evaluation

The weight and height of the patients were measured using a calibrated scale after requesting the patients to remove their shoes and any heavy clothing. Venous blood samples were obtained in the morning after the patients had fasted overnight for 12 h. Laboratory evaluations in all patients included determination of the blood cell counts, and measurement of the serum levels of AST, ALT, γ-glutamyl transpeptidase (GGT), cholinesterase (ChE), albumin, total cholesterol, low-density lipoprotein (LDL) cholesterol, high-density lipoprotein (HDL) cholesterol, triglyceride, fasting immunoreactive insulin (IRI), hyaluronic acid and type IV collagen 7s domain, and fasting plasma glucose. All of the parameters were measured using standard techniques.

Based on the previous study, the upper normal limit of the serum ALT was set at 40 IU/l [25, 26]. The FIB-4 index was calculated as age × AST (IU/l)/platelet count ($\times 10^9$/l)/$\sqrt{}$ALT (IU/l) [21]. The NAFLD fibrosis score was calculated according to the following formula: $-1.675 + 0.037 \times$ age (years) $+ 0.094 \times$ BMI (kg/m^2) $+ 1.13 \times$ impaired fasting glycemia or diabetes (yes = 1, no = 0) $+ 0.99 \times$ AST/ALT ratio $- 0.013 \times$ platelet ($\times 10^9$/l) $- 0.66 \times$ albumin (g/dl) [19]. The BARD score was estimated as the weighted sum of three variables (BMI >28 = 1 point, AST/ALT ratio >0.8 = 2 points, diabetes = 1 point) [20]. AAR was calculated as AST/ALT [18].

Histologic evaluation

All patients enrolled in this study had undergone a percutaneous liver biopsy under ultrasound guidance. Fatty liver was defined as the presence of >5 % steatosis, while steatohepatitis was defined as the presence of steatosis, inflammation, and hepatocyte ballooning [27–29]. The degree of steatosis was assessed based on the percentage of hepatocytes containing macrovesicular fat droplets, as follows: grade 0, no steatosis; grade 1, 5–33 % hepatocytes containing macrovesicular fat droplets; grade 2, 33–66 % hepatocytes containing macrovesicular fat droplets; grade 3, >66 % hepatocytes containing macrovesicular fat droplets. The individual parameters of fibrosis were scored independently according to the NASH Clinical Research Network (CRN) scoring system developed by the NASH CRN [30]. Advanced fibrosis was classified as stage 3 or 4 (bridging fibrosis or cirrhosis).

Statistical analysis

Statistical analysis was conducted using SPSS, version 12.0 (SPSS, Inc., Chicago, IL, USA). Continuous variables were expressed as mean ± standard deviation (SD). Qualitative data were represented as numbers, with the percentages indicated within parentheses. The statistical significances of differences in the quantitative data were determined using the t test or Mann-Whitney's U test. Because the variables were often not normally distributed, group comparisons of more than two independent groups were performed using the Kruskal-Wallis test. The percentage of cases with advanced fibrosis was compared between the ALT ≤40 and ALT >40 groups using Fisher's exact test. The diagnostic performances of the scoring systems were assessed by analyzing the receiver-operating characteristic (ROC) curves. The probabilities of a true-positive (sensitivity) and true-negative (specificity) assessment were determined for selected cutoff values, and the area under the ROC curve (AUROC) was calculated for each index. The Youden index was used to identify the optimal cutoff points. Differences were considered to be statistically significant at $p < 0.05$.

Results

Patient characteristics

Using a multicenter database, 1,102 biopsy-proven cases of NAFLD were investigated. Of these, the serum ALT levels were more than 40 IU/l in 867 (78.7 %) patients and less than or equal to 40 IU/l in 235 (17.4 %) patients. In NAFLD patients with serum ALT levels ≤40 IU/l, steatosis grade, inflammatory activity, and fibrosis stage were not correlated with the serum ALT levels ($p = 0.4536$, 0.6238, and 0.1158 respectively by Kruskal-Wallis analysis). The distribution of histological fibrosis stage in various serum ALT levels (≤40, 41–60, 61–80, 81–100, and ≥101 IU/l) is shown in Table 1. The distribution of the fibrosis stage in ALT level ≤40 was as follows: stage 0, $n = 91$ (38.7 %); stage 1, $n = 65$ (27.7 %); stage 2, $n = 41$ (17.4 %); stage 3, $n = 21$ (8.9 %); stage 4, $n = 17$ (7.2 %). The ratio of advanced fibrosis was 16.1 % (ALT ≤40 IU/l), 24.5 % (ALT 41–60 IU/l), 16.2 % (ALT 61–80 IU/l), 27.9 % (ALT 81–100 IU/l), and 25.0 % (≥101 IU/l) (Table 1). The percentage of cases with advanced fibrosis among NAFLD patients with serum ALT levels ≤40 IU/l was

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

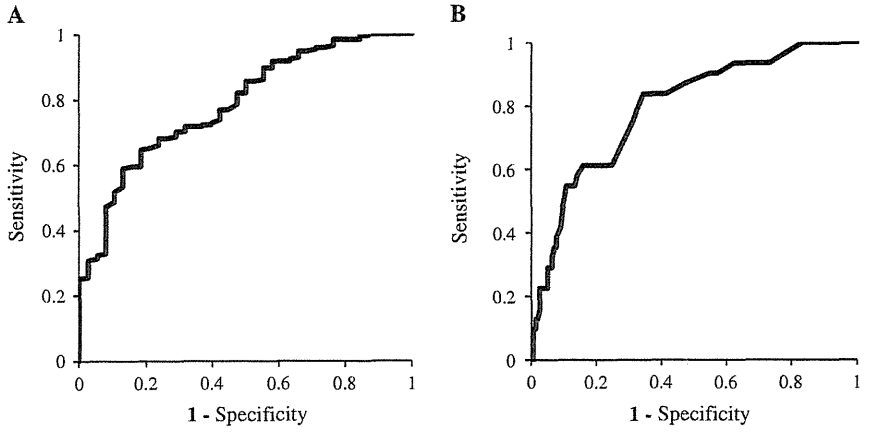

Fig. 1 Receiver-operating characteristic (ROC) curves for detecting advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** The platelet count, **b** type IV collagen 7s

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,

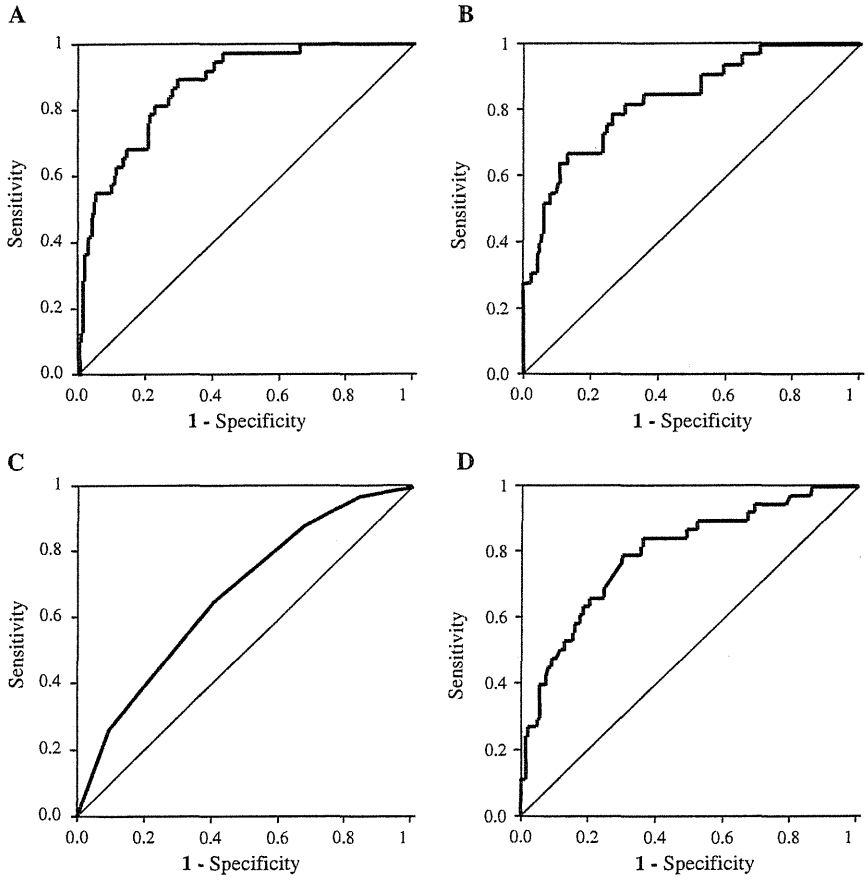

Fig. 2 Receiver-operating characteristic (ROC) curves for the noninvasive scores for a diagnosis of advanced fibrosis (stage 3 and 4) in NAFLD patients with serum ALT values ≤40 IU/l. **a** FIB-4 index, **b** NAFLD fibrosis score, **c** BARD score, **d** AST/ALT ratio (AAR)

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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Conflict of interest The authors have no conflicts of interest to disclose.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

Clinicopathological features of liver injury in patients with type 2 diabetes mellitus and comparative study of histologically proven nonalcoholic fatty liver diseases with or without type 2 diabetes mellitus

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Abstract
Background The Japan Society of Diabetes Mellitus reported that the leading cause of death in patients with diabetes mellitus (DM) was chronic liver disease; however, there are limited studies investigating the cause of liver injury in these patients. Our study aimed to clarify the clinicopathological features of liver injury and the characteristics of nonalcoholic fatty liver disease (NAFLD) in DM patients.
Methods In total, 5,642 DM patients and 365 histologically proven NAFLD patients were enrolled. Clinical and laboratory parameters and liver biopsy results were, respectively, recorded and analyzed for the two sets of patients.
Results Positivity rates for Hepatitis B surface antigens (HBsAg) and anti-hepatitis C virus antibodies (anti-HCV Ab) were 1.7 and 5.1 %, respectively. The proportion of drinkers consuming 20–59 g and ≥60 g alcohol daily was 14.9 and 4.3 %, respectively. The percentage of DM patients with elevated serum alanine aminotransferase (ALT) levels (≥31 IU/L) was 28.6 %. Alcohol consumption had no significant effect on serum ALT levels. Seventy-two percent of HBsAg-positive patients were serum hepatitis B virus (HBV)-DNA negative, whereas 10 % exhibited high levels of the same (>4.0 log copies/ml).

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Thirty-eight percent of anti-HCV Ab-positive patients were serum HCV-RNA negative. Among the NAFLD patients, the frequencies of NASH and advanced stage NASH were significantly higher in male DM patients than in male patients without DM.

Conclusions Although HBsAg- and anti-HCV Ab-positivity rates were high in our Japanese DM patients, a majority of liver injuries could be associated with NAFLD/nonalcoholic steatohepatitis.

Keywords Nonalcoholic fatty liver disease · Nonalcoholic steatohepatitis · Diabetes mellitus · Hepatitis virus carrier · Alcoholic liver disease · Nationwide study

Abbreviations

HCC	Hepatocellular carcinoma
NAFLD	Nonalcoholic fatty liver disease
DM	Diabetes mellitus
NASH	Nonalcoholic steatohepatitis
HBV	Hepatitis B virus
HCV	Hepatitis C virus
AST	Aspartate aminotransferase
ALT	Alanine aminotransferase
GGT	Gamma glutamyl transpeptidase
FPG	Fasting plasma glucose
HOMA-IR	The homeostasis model assessment of insulin resistance index
HBsAg	Hepatitis B surface antigen
anti-HBc Ab	Anti-hepatitis B core antibody
anti-HCV Ab	Anti-hepatitis C virus antibody
HBV-DNA	Hepatitis B virus-deoxyribonucleic acid
HCV-RNA	Hepatitis C virus-ribonucleic acid
OR	Odds ratio
CI	Confidence interval

Introduction

As per the International Diabetes Federation, the number of diabetes mellitus (DM) sufferers rose to 366 million in 2011, representing 8.3 % of the global adult population, which is increasing in every country [1]. Worldwide, hepatocellular carcinoma (HCC) is the fifth most common cancer and the third most common cause of cancer mortality [2]. HCC largely occurs in patients with chronic liver disease. Persistent hepatitis C virus (HCV) or hepatitis B virus (HBV) infections are the main causes of HCC; however, non-HCV- and non-HBV-associated HCC cases are increasing in Japan [3].

In 2007, the Japan Society of DM reported that the most frequent cause of death among 18,385 DM patients who died in hospitals during 1991–2000 was malignancy (34.1 %), followed by ischemic heart disease (10.2 %) and cerebrovascular disease (9.8 %) [4]. Among the malignancies, HCC showed the highest frequency (8.6 %), followed by lung (5.3 %), pancreatic (4.8 %), and gastric cancer (3.5 %). Furthermore, the frequency of deaths caused by liver cirrhosis was 4.7 %, and in total, 13.3 % DM patients died of liver diseases. The cancer death rate in that study was quite different from that reported in the general Japanese population, in which lung (5.7 %), gastric (4.7 %), and colon (2.5 %) cancer occur with high frequencies [5]. Moreover, the death rate from liver diseases (13.3 %) was three times higher than that in the general Japanese population (HCC 3.2 %, liver cirrhosis 1.5 %, total 4.7 %) [6]. However, the incidences of HBV and HCV infection and the details of alcohol intake were not analyzed in that report.

The Japan Nonalcoholic Steatohepatitis (NASH) Study Group was founded in 2007 to investigate the cause of death in DM patients, the genetic factors in nonalcoholic fatty liver disease (NAFLD) patients, and the background of NASH-HCC patients [7]. This study focused on clarifying the cause of liver injury in Japanese DM patients and investigating the histological distribution of NAFLD in patients with and without DM.

Patients and methods

Patients

In total, 5,642 DM patients (3,238 males, 2,404 females) who visited nine DM clinics belonging to the Japan NASH Study Group (Saiseikai Suita Hospital; Kagoshima University Graduate School of Medical and Dental Sciences; Graduate School of Medicine, The University of Tokyo; Kanazawa University Graduate School of Medical Science; Department of Medicine, Asahikawa Medical College; Yamagata University Faculty of Medicine; Kyoto Prefectural University of Medicine; Okayama Saiseikai General Hospital; Fukui-ken Saiseikai Hospital) between January 2008 and December 2009 were enrolled in this observational study.

Three hundred and sixty-five NAFLD patients (182 males, 183 females) who visited Saiseikai Suita Hospital were enrolled in the histopathological study.

The study protocol was approved by the Human Ethics Committee of each participating hospital. Informed consent was obtained from all patients in accordance with the Declaration of Helsinki.

Clinical and laboratory assessment

Demographic parameters, including age, sex, height, weight, and body mass index (BMI), and comorbidities, including alcohol consumption, hypertension, and dyslipidemia, were

recorded for all subjects in addition to the treatment administered for DM and the frequency of HCC occurrence. Clinical laboratory tests were conducted to measure aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyl transpeptidase (GGT), albumin, total cholesterol, triglyceride (TG), ferritin, uric acid, hemoglobin A1c, fasting plasma glucose (FPG), and insulin levels. The homeostasis model assessment of insulin resistance (HOMA-IR) index; platelet (PLT) count; and hyaluronic acid, type IV collagen 7S, hepatitis B surface antigen (HBsAg), anti-hepatitis B core antibody (anti-HBc Ab), anti-HCV antibody (anti-HCV Ab), HBV-DNA, and HCV-RNA levels were also measured.

Blood samples were procured in the morning after overnight fasting. HOMA-IR was only calculated for subjects with FPG <140 mg/dL. HBV-DNA levels were measured by PCR (Amplicor HBV-DNA kit, Roche Diagnostics) or real-time PCR (TaqMan HBV-DNA kit, Roche Diagnostics) for HBsAg-positive, whereas HCV-RNA levels were measured by PCR (Amplicor HCV-RNA kit, version 2.0, Roche Diagnostics) or real-time PCR (TaqMan HCV-RNA kit, Roche Diagnostics) for anti-HCV Ab-positive patients.

Histopathological examination

In total, 365 patients (177 non-DM and 188 DM) at Saiseikai Suita Hospital fulfilled the criteria for NAFLD, namely serum HBsAg and anti-HCV Ab negativity, no alcohol consumption, and the absence of autoimmune liver diseases or hereditary liver injury. These patients underwent an ultrasound-guided liver biopsy using a 16G needle.

Specimens were fixed in formalin, embedded in paraffin, and subjected to hematoxylin–eosin, Masson trichrome, and Perl's iron staining. Histological features of samples were interpreted according to a method described by Matteoni et al [8]. NASH stage was classified according to Brunt's classification [9].

Statistical analysis

All statistical analyses were performed using SPSS for Windows (SPSS Japan Inc.). Data were summarized by frequency for categorical variables and mean ± standard deviation (SD) for continuous variables. The Chi-square test was used to determine the differences between categorical variables. Student's t test was used to compare means of continuous valuables with equal variance, and the Mann–Whitney U test was used for non-normally distributed variables. The Cochran–Armitage test was used to study the trend of continuous variables. Forward stepwise logistic regression analysis was used to identify independent variables related to elevated serum ALT (≥31 IU/L) levels. A p value of <0.05, obtained by a two-tailed test, was considered statistically significant.

Since there is no official report on the HBV and HCV carrier rate in the general Japanese population, we utilized blood donor data for comparison with our patients [10].

Results

Baseline characteristics

The mean age and BMI of male and female DM patients was 62.2 and 64.8 years and 24.5 and 24.7 kg/m^2, respectively (Table 1). Hypertension and dyslipidemia occurred in 51.0 and 63.3 % of DM patients, respectively. Respective DM treatment types in DM patients were as follows: no medication, 20.5 %; oral drugs, 47.7 %; insulin, 28.9 %; and oral drugs and insulin, 2.8 % (Table 2).

Mean ALT level was significantly higher in males (30.6 IU/L) than in females (Table 1). Abnormal serum ALT levels (≥31 IU/L) were found in 28.6 % of DM patients (males 32.8 %, females 23.0 %). When the healthy upper limit of abnormal serum ALT level in females was defined as 20 IU/L according to Prati et al.'s [11] criteria, the frequency of abnormal ALT (≥21 IU/L) levels in females was 43 %. The mean PLT count was 20.8 × 10^4/μL in males and 21.9 × 10^4/μL in females. Mean values of other clinical laboratory tests are shown in Table 1.

Prevalence of HBV and HCV infection and drinking and their effects on liver function tests

HBsAg positivity was detected in 1.7 % of DM patients (M 1.8 %, F 1.6 %) (Table 2); this was significantly higher than that (total 0.9 %, M 1.0 %, F 0.7 %) detected in 1.7 million blood donors aged >40 years ($p < 0.001$). For both sexes, the HBsAg detection rate was significantly higher in DM patients than in blood donors in the 50- to 59- and 60- to 69-year age groups ($p < 0.05$) (Fig. 1). There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes.

Seventy-two percent of HBsAg-positive patients (M 69 %, F 79 %) demonstrated HBV-DNA negativity (<2.6 log copies/ml) (Table 3). Of the HBsAg-positive patients, only 10 % showed high serum HBV-DNA levels (≥4.0 log copies/ml); these could be HBV infection-induced liver injury cases. Mean values of age, serum ALT level, and PLT counts in HBV-DNA-negative HBV carriers were 63.6 years, 25.3 IU/L, and 20.5 × 10^4/μL, respectively. HBV-DNA-negative HBV carriers were older and exhibited lower ALT levels and higher PLT counts; however, the differences were not significant.

Table 1 Backgrounds of diabetes mellitus patients (1)

Characteristic	Total subjects		Males		Females		
	n	M ± SD	*n*	M ± SD	*n*	M ± SD	*p*
Age (years)	5,510	63.3 ± 12.7	3,164	62.2 ± 12.5	2,346	64.8 ± 12.9	<0.001
BMI (kg/m^2)	5,173	24.6 ± 4.7	2,987	24.5 ± 4.2	2,186	24.7 ± 5.2	0.629
Aspartate aminotransferase (IU/L)	5,568	26.4 ± 17.2	3,188	27.1 ± 18.0	2,380	25.5 ± 15.9	<0.001
Alanine aminotransferase (IU/L)	5,569	28.2 ± 24.5	3,190	30.6 ± 26.9	2,379	24.9 ± 20.5	<0.001
GGT (IU/L)	5,476	48.3 ± 72.5	3,131	59.6 ± 86.7	2,345	33.1 ± 42.9	<0.001
Albumin (g/dL)	5,031	4.2 ± 0.4	2,869	4.2 ± 0.5	2,162	4.1 ± 0.4	<0.001
Platelet ($\times 10^4/\mu L$)	5,419	21.3 ± 6.1	3,112	20.8 ± 6.0	2,307	21.9 ± 6.1	<0.001
Fasting plasma glucose (FPG; mg/dL)	5,123	152.7 ± 61.7	2,945	156.0 ± 63.9	2,178	148.3 ± 58.2	<0.001
HbA1c (%)	5,479	7.2 ± 1.7	3,143	7.2 ± 1.7	2,336	7.2 ± 1.6	0.744
HOMA-IR (FPG <140)	1,005	2.55 ± 2.60	570	2.51 ± 2.59	435	2.61 ± 2.60	0.209
Total cholesterol (mg/dL)	5,260	195.1 ± 39.5	3,016	191.6 ± 40.0	2,244	199.6 ± 38.5	<0.001
Triglycerides (mg/dL)	5,443	136.3 ± 102.7	3,119	145.1 ± 111.9	2,324	124.5 ± 87.5	<0.001
Hyaluronic acid (ng/mL)	559	74.5 ± 98.6	319	59.3 ± 73.0	240	94.6 ± 122.1	<0.001
Type 4 collagen 7S (ng/mL)	474	4.9 ± 2.0	269	4.8 ± 2.0	205	4.9 ± 1.9	0.544
Ferritin (ng/mL)	1,838	142.0 ± 157.0	1,084	171.9 ± 174.9	754	99.1 ± 114.1	<0.001
Uric acid (mg/dL)	3,645	5.4 ± 1.5	2,043	5.7 ± 1.4	1,602	4.9 ± 1.4	<0.001

Results are shown as mean ± SD

GGT gamma glutamyl transpeptidase, *HOMA-IR* homeostasis model assessment of insulin resistance

Table 2 Backgrounds of diabetes mellitus patients (2)

Characteristic	Total subjects		Males		Females		
	n	Positive (%)	*n*	Positive (%)	*n*	Positive (%)	*p*
Hepatitis B surface antigen	4,867	83 (1.7 %)	2,796	50 (1.8 %)	2,071	33 (1.6 %)	0.604
Anti-hepatitis B core antibody	3,211	958 (29.8 %)	1,824	572 (31.4 %)	1,387	386 (27.8 %)	0.030
Anti-hepatitis C virus antibody	4,877	247 (5.1 %)	2,812	144 (5.1 %)	2,065	103 (5.0 %)	0.834
Alcohol	4,443		2,554		1,889		<0.001
<20 g/day		3589 (80.8 %)		1769 (69.3 %)		1820 (96.3 %)	
20–59 g/day		661 (14.9 %)		609 (23.8 %)		52 (2.8 %)	
≥60 g/day		193 (4.3 %)		176 (6.9 %)		17 (0.9 %)	
Hypertension	4,936	2515 (51.0 %)	2,841	1419 (49.9 %)	2,095	1096 (52.3 %)	0.100
Dyslipidemia	5,423	3434 (63.3 %)	3,091	1882 (60.9 %)	2,332	1552 (66.6 %)	<0.001
Diabetes mellitus (intervention)	5,227		3,013		2,214		0.002
None		1072 (20.5 %)		629 (20.9 %)		443 (20.0 %)	
Oral drugs		2495 (47.7 %)		1489 (49.4 %)		1006 (45.4 %)	
Insulin		1513 (28.9 %)		810 (26.9 %)		703 (31.8 %)	
Oral drugs + insulin		147 (2.8 %)		85 (2.8 %)		62 (2.8 %)	
Hepatocellular carcinoma	4,700	67 (1.4 %)	2,696	48 (1.8 %)	2,004	19 (0.9 %)	0.017

Anti-HCV Ab positivity was detected in 5.1 % (M 5.1 %, F 5.0 %) of DM patients; this rate was significantly higher than that (total 1.0 %, M 1.1 %, F 1.0 %) in blood donors of every age group of both sexes ($p < 0.001$), except for females aged 50–59 years (Table 2; Fig. 2). ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts ($p < 0.001$, $p < 0.05$) (Fig. 2). For both sexes,

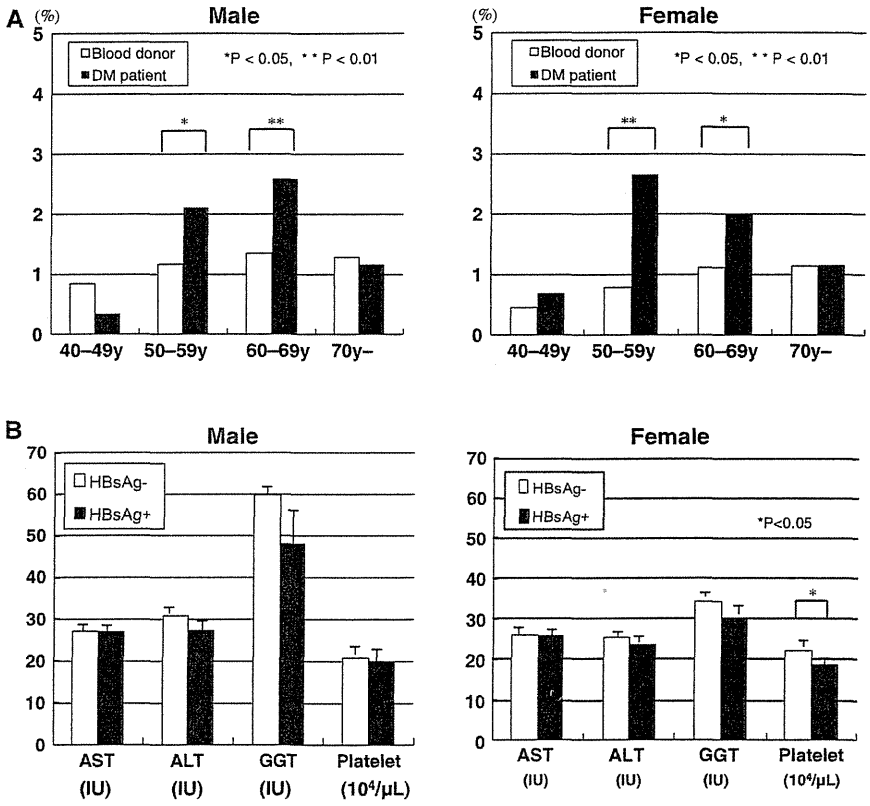


Fig. 1 Prevalence of HBV infection and the effect of HBV infection on laboratory tests in DM patients. **a** Prevalence of HBV infection in blood donors and DM patients. **b** The effect of HBV infection on laboratory tests in DM patients. There were no significant differences in serum AST, ALT, and GGT levels between HBsAg-positive and HBsAg-negative DM patients of both sexes. *Error bars* SD

Table 3 Serum HBVDNA and HCVRNA levels, age, serum ALT level, and platelet (PLT) counts in HBsAg-positive patients and anti-HCV Ab-positive patients

	% (*n*)	Mean age (years)	Mean ALT levels (IU/L)	Mean PLT count($\times 10^4$/μL)
Serum HBV-DNA[a]				
Negative (<2.6 log copy/ml)	72 (29)	63.6	25.3	20.5
Positive (=2.6 log copy/ml)	28 (11)	55.6	28.0	18.5
=2.6<4.0	18 (7)	61.9	26.6	18.9
=4.0	10 (4)	50.0	30.5	17.8
Serum HCV-RNA[b]				
Negative (<2.7 log IU/ml)	38 (57)	67.2	28.2	17.7
Positive (=2.7 log IU/ml)	62 (91)	67.4	51.7	15.3
=2.7<5.0	3 (4)	65.1	28.0	16.4
=5.0	59 (87)	67.5	52.7	15.2

[a] Results are presented as either frequency or mean in 40 HBsAg-positive patients

[b] Results are presented as either frequency or mean in 148 anti-HCV Ab-positive patients

the PLT count was significantly lower in anti-HCV Ab-positive DM patients than in their negative counterparts ($p < 0.001$).

Thirty-eight percent of anti-HCV Ab-positive patients (M 36 %, F 42 %) demonstrated HCV-RNA negativity (Table 3), and 96 % of HCV-RNA-positive patients exhibited high serum HCV-RNA levels (≥5.0 log IU/ml). Serum ALT levels in anti-HCV Ab-positive patients with HCV-RNA positivity and those with HCV-RNA negativity were 51.7 ± 39.7 and 28.2 ± 18.1 IU/L, respectively, whereas those in anti-HCV Ab-negative patients were 27.7 ± 22.8 IU/L. Serum ALT levels were significantly higher in HCV-RNA-positive patients than in HCV-RNA-negative patients ($p < 0.001$).

The proportion of DM patients consuming >60 g and 20–59 g alcohol daily was 4.3 % (M 6.9 %, F 0.9 %) and

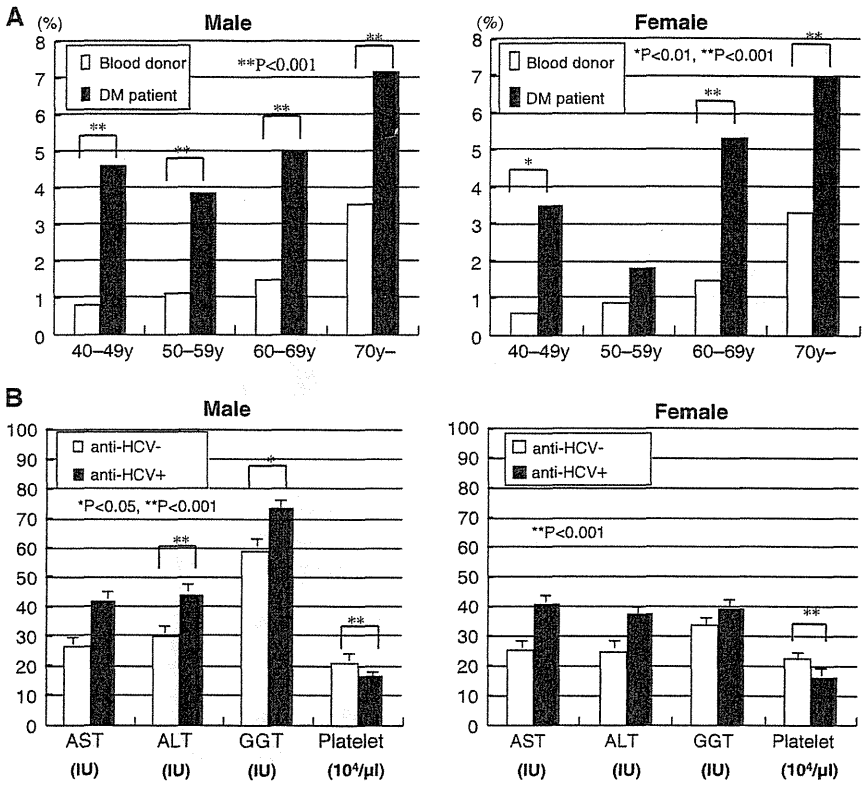


Fig. 2 Prevalence of HCV infection and the effect of HCV infection on laboratory tests in DM patients. **a** Prevalence of HCV infection in blood donors and DM patients. **b** The effect of HCV infection on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. ALT and GGT levels were significantly higher in male anti-HCV Ab-positive patients than in their negative counterparts. *Error bars* SD

14.9 % (M 23.8 %, F 2.8 %), respectively (Table 2). The highest percentage of drinkers were males in the 60- to 69-year age group and females in the <40-year age group. Male drinkers consuming >60 g alcohol daily had significantly higher serum AST and GGT levels compared with nondrinkers (patients consuming <20 g of daily alcohol intake) ($p < 0.001$). Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. Drinkers of both sexes consuming 20–59 g alcohol daily had significantly higher serum GGT levels ($p < 0.001$) (Fig. 3).

Factors related to serum ALT levels

With increasing age in both sexes, the number of DM patients with elevated serum ALT levels and high BMI decreased, whereas those with decreased PLT counts increased. The number of DM patients with elevated serum ALT levels increased with increasing BMI in both sexes (Fig. 4).

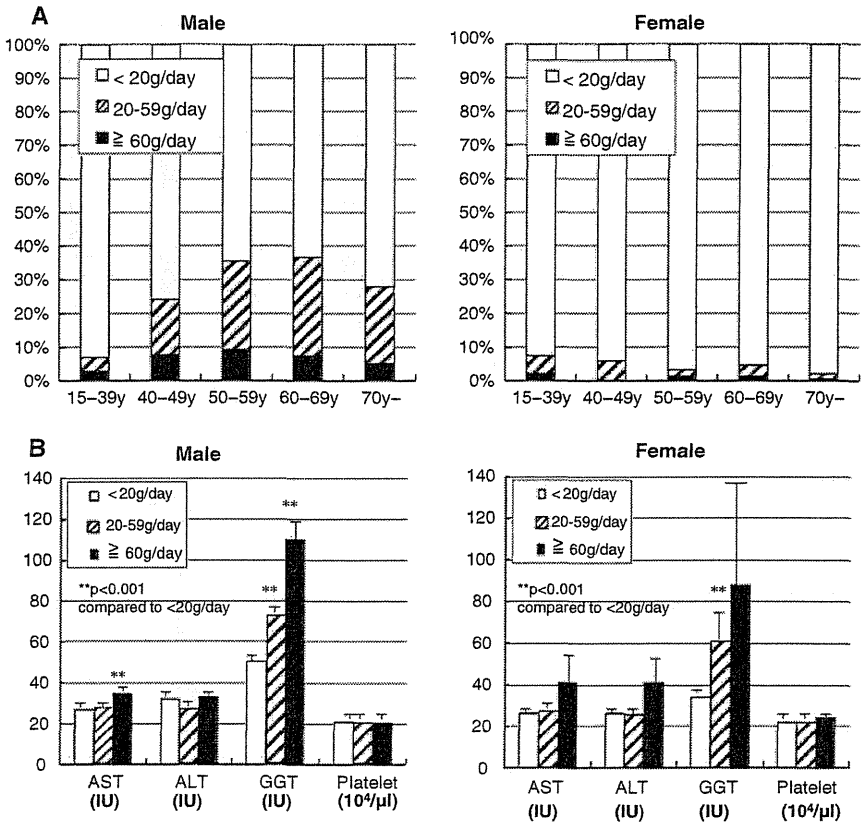
A forward stepwise logistic regression model yielding odds ratios (ORs) and 95 % confidence intervals (CIs) was used to analyze the factors related to elevated serum ALT levels. The model included BMI, age, drinking status, HBsAg status, anti-HCV Ab status, PLT count, hypertension status, and dyslipidemia status as independent variables. The odds ratio shown indicates the change in odds for one SD increase in each variable.

Multivariate analysis showed that age (M: OR 0.674, CI 0.613–0.741; $p < 0.001$; F: OR 0.767, CI 0.683–0.861; $p < 0.001$), PLT count (M: OR 0.806, CI 0.732–0.886; $p < 0.001$, F: OR 0.714, CI 0.632–0.808, $p < 0.001$), anti-HCV Ab status (M: OR 1.321, CI 1.218–1.433; $p < 0.001$; F: OR 1.232, CI 1.117–1.359; $p < 0.001$), and BMI (M: OR 1.509, CI 1.374–1.657; $p < 0.001$; F: OR 1.487, CI 1.330–1.663; $p < 0.001$) were significantly associated with elevated serum ALT levels (Table 4).

For both sexes, AST and ALT levels were similar in drinkers consuming 20–59 g alcohol daily and those consuming <20 g alcohol daily (Fig. 3). After eliminating HBV-positive patients and/or HCV carriers and heavy drinkers consuming >60 g alcohol daily, the number of male, female, and total DM patients with elevated serum ALT levels were 33.4, 23.3, and 28.3 %, respectively. These values were comparable with those in all DM patients, including those with hepatitis and/or those consuming alcohol (M 32.8 %, F 23.0 %, total 28.6 %).

Liver histology in DM patients

The median age of histologically proven, DM- ($n = 87$) and non-DM-associated ($n = 95$) male NAFLD patients



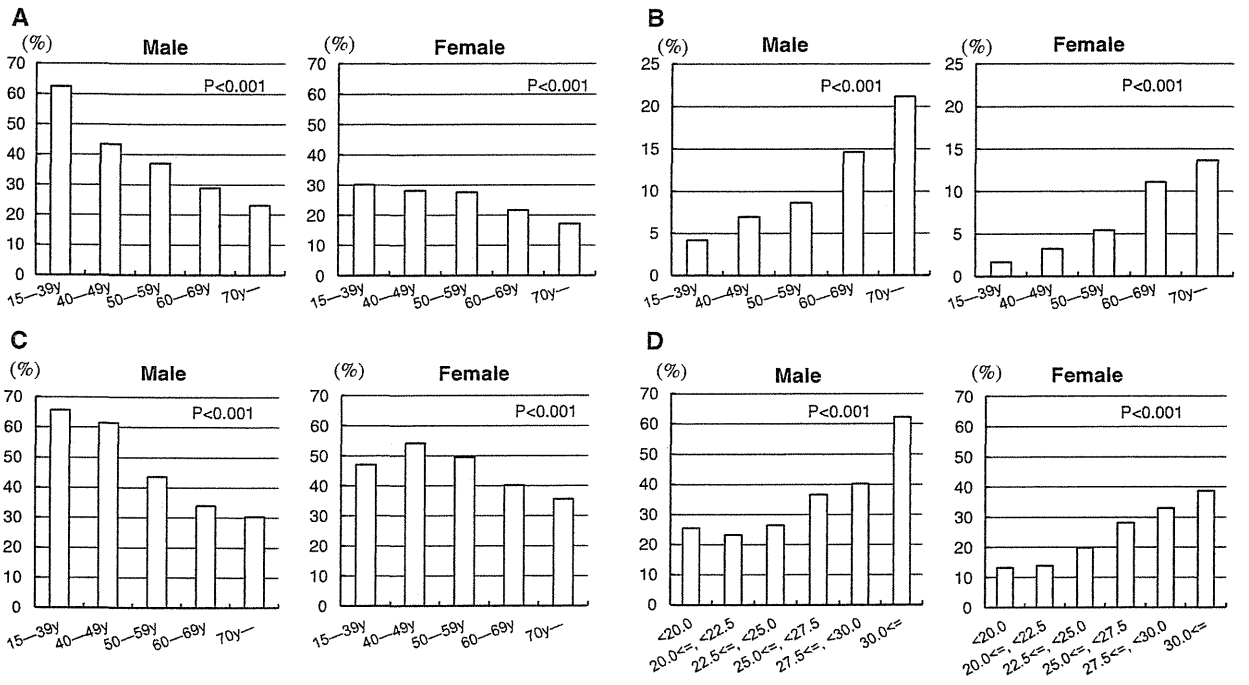


Fig. 3 Drinking habits and the effect of alcohol consumption on laboratory tests in DM patients. **a** Drinking habits in individual age. **b** The effect of alcohol consumption on laboratory tests in DM patients. *GGT* gamma glutamyl transpeptidase. Serum ALT levels in drinkers consuming >60 g alcohol daily were comparable with those in nondrinkers. *Error bars* SD

Fig. 4 Influence of age on the ratio of patients with elevated serum ALT level, decreased PLT count and abnormal BMI, and the relationship between BMI and the ratio of patients with elevated serum ALT level. **a** The ratio of patients with elevated serum ALT level (≥31 IU/L). **b** The ratio of patients with decreased PLT count (<15 × 10^4/μL). **c** The ratio of patients with abnormal BMI (≥25). **d** The relationship between BMI and the ratio of patients with elevated serum ALT level (≥31 IU/L)

Table 4 Multivariate analysis to identify independent variables related to elevated serum ALT level (≥31 IU/L)

	Regression coefficient	Standard error	Odds ratio	95 % confidence interval	*p*
Males					
Age	−0.394	0.048	0.674	0.613–0.741	<0.001
Platelet	−0.216	0.049	0.806	0.732–0.886	<0.001
Anti-hepatitis C virus	0.278	0.042	1.321	1.218–1.433	<0.001
Body mass index	0.411	0.048	1.509	1.374–1.657	<0.001
Females					
Age	−0.265	0.059	0.767	0.683–0.861	<0.001
Platelet	−0.336	0.063	0.714	0.632–0.808	<0.001
Anti-hepatitis C	0.208	0.050	1.232	1.117–1.359	<0.001
Body mass index	0.397	0.057	1.487	1.330–1.663	<0.001

was 60 and 45 years, respectively; in corresponding females, the median age was 66 ($n = 101$) and 61 years ($n = 82$), respectively. No significant difference was noted in BMI between DM and non-DM NAFLD patients (M 26.0 and 27.5 kg/m^2, respectively; F 26.0 and 27.0 kg/m^2, respectively). Male NAFLD patients without DM were significantly younger than those with DM ($p < 0.001$).

NAFLD patients were classified according to Matteoni's classification. Type 1, 2, 3, and 4 cases were 14 (16 %), 14 (16 %), 4 (5 %), and 55 (63 %), respectively, among male DM patients and 28 (29 %), 23 (24 %), 11 (12 %), and 33 (35 %), respectively, among male non-DM patients. Type 1, 2, 3, and 4 cases were 16 (16 %), 8 (8 %), 4 (4 %), and 73 (72 %), respectively, among female DM patients and 9 (11 %), 9 (11 %), 7 (9 %), and 57 (69 %), respectively, among female non-DM patients (Fig. 5). The frequency of Type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$).The rate of Type 4 NASH was high in both female DM and non-DM patients.

In total, 244 (M 103, F 141) NASH patients were classified according to Brunt's classification. The number of patients with stage 0 (Matteoni Type 3), 1, 2, 3, and 4 were 2 (3 %), 27 (46 %), 10 (17 %), 17 (29 %), and 3 (5 %), respectively, among male DM patients and 10 (23 %), 16 (36 %), 8 (18 %), 9 (21 %), and 1 (2 %), respectively, among male non-DM patients. Stage 0, 1, 2, 3, and 4 cases were 4 (5 %), 25 (33 %), 24 (31 %), 13 (17 %), and 11 (14 %), respectively, among female DM patients and 4 (6 %), 28 (44 %), 20 (31 %), 10 (16 %), and 2 (3 %), respectively, among female non-DM patients (Fig. 6). The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$).The rate of Stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$).

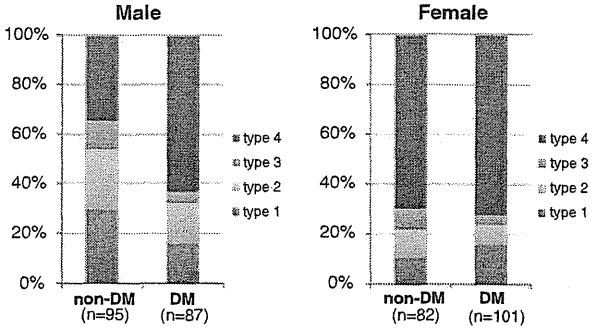


Fig. 5 Distribution of Matteoni's type classification in individual status of glucose metabolism among NAFLD patients. The frequency of type 4 NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.001$). The rate of type 4 NASH was high in both female DM and non-DM patients

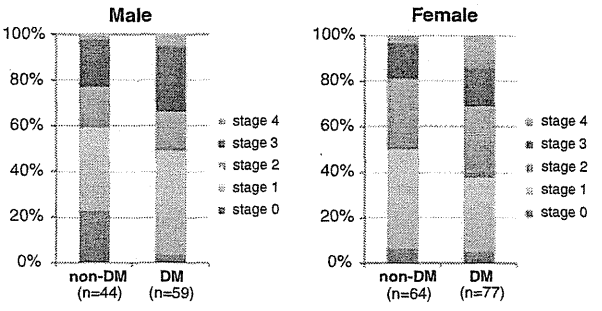


Fig. 6 Distribution of Brunt's stage in individual status of glucose metabolism among NASH patients. The frequency of advanced stage NASH was significantly higher in male DM patients than in male non-DM patients ($p < 0.05$). The rate of stage 4 NASH was higher in female DM patients than in female non-DM patients; however, the difference was not significant ($p = 0.198$)

HCC incidence in DM patients

In total, 67 (M 48, F 19) HCC cases (1.4 %) were reviewed (Table 2). HCC incidence was significantly higher in males

than in females. Five of 67 HCC patients consumed >60 g alcohol daily, and two of these five patients were anti-HCV Ab positive. HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence in the HCC patients was 8.6, 50.0, and 41.4 %, respectively. In a Japanese nationwide survey of 19,499 HCC patients [3], HBsAg positivity, anti-HCV Ab positivity, and non-B non-C prevalence was 15.0, 67.7, and 17.3 %, respectively. Non-B non-C prevalence was higher in our DM patients with HCC than in the nationwide HCC survey participants ($p < 0.001$). Mean PLT count in DM patients with HCC was as follows: HBsAg-positive patients, 12.4 ± 6.8; anti-HCV Ab-positive patients, 12.4 ± 5.6; and non-B non-C patients, 16.0 ± 7.0 ($\times 10^4/\mu L$); PLT count was significantly higher in the non-B non-C patients than in the anti-HCV-positive patients ($p < 0.05$). Mean BMI in these three patient groups was as follows: HBsAg-positive patients, 23.2 ± 5.1; anti-HCV Ab-positive patients, 22.8 ± 3.3; and non-B non-C patients, 27.2 ± 4.4 (kg/m^2); BMI was significantly higher in the non-B non-C patients than in the anti-HCV Ab-positive patients ($p < 0.001$).

Discussion

This is the first multicenter study, as per our knowledge, that clarifies the cause of liver injury in DM patients in Japan. Most Japanese HBV carriers are genotype C, acquired via perinatal vertical transmission or early childhood infection [12]. The HBV carrier rate in Japan is higher than that in western countries and significantly lower than that in other Asian countries [13]. In 1986, the Japanese government initiated a nationwide hepatitis B immunization program for infants born to HBV carrier mothers to prevent perinatal transmission. Consequently, the number of young serum HBsAg-positive individuals is extremely low. In our study, although the HBV carrier rate in DM patients was significantly higher than that in blood donors, 72 % of HBsAg-positive patients were serum HBV-DNA negative. Only 10 % of HBsAg-positive patients exhibited high serum HBV-DNA levels (≥4.0 log copies/ml), which is likely to induce hepatitis. These results indicate that a majority of DM patients who are HBV carriers may be asymptomatic.

Chronic hepatitis C may result in life-threatening complications, including cirrhosis and HCC. Worldwide, cirrhosis can be attributed to HBV (30 %) and HCV infection (27 %) [14]. The leading cause of cirrhosis among HBV and HCV sufferers and alcohol consumers varies with individual countries. A recent nationwide Japanese survey reported the etiology of cirrhosis in Japan as follows: HCV 60.9 %, HBV 13.9 %, alcoholism 13.6 %, primary biliary cirrhosis 2.4 %, NASH-related 2.1 %, and autoimmune hepatitis 1.9 % [15]. However, we must consider that hepatic triglycerides diminish with liver fibrosis progression in NASH patients (so-called "burned-out" NASH), resulting in difficulty in diagnosing NASH. Sixty-two percent of anti-HCV Ab-positive DM patients were HCV-RNA positive; these patients showed significantly higher serum ALT levels compared with HCV-RNA-negative patients. These results indicate that HCV infection is involved in the etiology of liver disease in DM patients.

There is no doubt that the positive rates of serum HBsAg and anti-HCV Ab in the general population are higher than in blood donors. Unfortunately, there were no data in the distribution of the rate of hepatitis virus carriers in each age group in Japan. In the present study, the positive rates of HBsAg and anti-HCV Ab in DM patients were significantly higher than that in blood donors. However, the present study demonstrated that most of HBsAg positive patients were negative for serum HBV DNA or had low serum HBV DNA levels and around one-third of anti-HCV Ab positive patients were negative for serum HCV RNA.

These results indicate the possibility that the frequency of hepatitis virus carriers in DM patients is higher than that in general population but no significant differences might be noted between DM patients and the general population.

Alcohol consumption is reportedly a significant factor associated with the risk of HCC development in patients with NASH-associated cirrhosis [16]. In our study, serum AST and ALT levels were comparable between drinkers consuming 20–59 g alcohol daily and nondrinkers. The ratio of heavy drinkers consuming >60 g alcohol daily was low (4.3 %) in our study. Moreover, drinking was not chosen as a variable related to elevated serum ALT levels. These results suggest that alcohol intake is not an important factor in the pathogenesis of liver disease in DM patients.

In our study, the frequency of anti-HCV Ab-positive DM patients was 5 %, whereas the serum HCV-RNA positivity rate in anti-HCV Ab-positive patients was 62 %. Therefore, the HCV carrier rate was calculated as 3 %. Since the proportion of HCV carriers and patients with elevated ALT levels were 3 % and up to 29 %, respectively, the influence of HCV infection is estimated to be no more than 10 % (3 % divided by 29 %) among DM patients with elevated ALT levels. There was no significant change in the number of DM patients with elevated ALT levels before and after elimination of HBV and/or HCV carriers and heavy drinkers. These results suggested that the major cause (up to 90 %) of liver injury in DM patients may be NAFLD.

In the present study, the frequency of advanced stage NASH was significantly higher in male DM patients than

in male non-DM patients. Neuschwander-Tetri et al. [17] reported that patients with advanced stage NASH were more likely to have DM. Mayaaki et al. [18] also examined the relationship between hepatic fibrosis stage and DM prevalence. In the mild fibrosis group, only 42 % were complicated with DM, whereas in the severe fibrosis group, the prevalence was as high as 71 % ($p = 0.020$). Lo et al. [19] reported that DM exacerbated diet-induced NASH fibrosis in mice. Therefore, DM may be an important factor in hepatic fibrosis development in NAFLD patients.

HCC frequency is significantly higher in obese and DM patients than in non-obese and non-DM patients [20, 21]. Recently, Tokushige et al. [22] reported on the backgrounds of Japanese HCC patients, and non-B non-C HCC accounted for 16 % of cases. A recent report has shown that NASH patients are likely to develop HCC in an earlier stage of fibrosis compared with chronic hepatitis C patients [23]. Our previous study analyzed 87 histologically proven NASH-HCC patients [24]; 37 % (20/54) of male HCC patients had a mild to moderate stage of liver fibrosis (F1 or F2); however, no female HCC patients were F1 stage, and only 15 % (5/33) were F2 stage. In the present study, DM patients with non-B non-C HCC exhibited a tendency to have higher PLT counts than those in DM patients with HCV-HCC, indicating that non-B non-C HCC is more likely to occur in DM patients with less advanced liver disease than in those with viral hepatitis.

In conclusion, HBsAg and anti-HCV Ab positivity rates were high; however, most of these patients were HBV-DNA negative or had low serum HBV-DNA levels. One-third of anti-HCV Ab-positive patients were HCV-RNA negative, and 4.3 % patients were drinkers whose ALT levels were comparable with those of nondrinkers. From these results, we conclude that up to 90 % of Japanese DM patients with liver injury may have NAFLD/NASH.

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Conflict of interest The authors declare that they have no conflicts of interest to disclose.

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