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# Fatty liver in men is associated with high serum levels of small, dense low-density lipoprotein cholesterol

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**Abstract**

**Aims:** Our study addressed potential associations between fatty liver and small, dense low-density lipoprotein cholesterol (sd-LDL-C) levels using a cross-sectional analysis.

**Methods:** We enrolled 476 male subjects. Serum sd-LDL-C concentrations were determined using precipitation assays.

**Results:** Subjects were divided into four groups based on triglyceride (TG) and LDL-C levels: A, TG < 150 mg/dl and LDL-C < 140 mg/dl; B, TG < 150 mg/dl and LDL-C ≥ 140 mg/dl; C, TG ≥ 150 mg/dl and LDL-C < 140 mg/dl; and D, TG ≥ 150 mg/dl and LDL-C ≥ 140 mg/dl. sd-LDL-C levels and the prevalence of fatty liver were significantly higher in groups B, C, and D than in group A. Subjects were also categorized into four groups based on serum sd-LDL-C levels; the prevalence of fatty liver significantly increased with increasing sd-LDL-C levels. Additionally, logistic regression analysis revealed an independent association between sd-LDL-C concentrations and fatty liver using such potential confounders as obesity and hyperglycemia as variables independent of elevated TG or LDL-C levels.

**Conclusions:** Fatty liver is a significant determinant of serum sd-LDL-C levels independent of the presence of obesity or hyperglycemia. Fatty liver may alter hepatic metabolism of TG and LDL-C, resulting in increased sd-LDL-C levels.

**Keywords:** Small dense low-density lipoprotein, Fatty liver, Type 2 diabetes mellitus, Metabolic syndrome

## Introduction

Atherogenic lipid profiles in patients with metabolic syndrome or glucose intolerance are characterized by hypertriglyceridemia, elevated apolipoprotein B levels, reduced high-density lipoprotein cholesterol (HDL-C) concentrations, and an increased proportion of small, dense low-density lipoprotein (sd-LDL) particles [1-3]. Compared with large LDL, sd-LDL particles show increased penetration of the arterial wall, lower affinity for the LDL receptor, longer half-life in plasma, greater susceptibility to glycation, and lower resistance to oxidative stress, suggesting that sd-LDL is highly atherogenic [4,5]. Indeed, patients with high levels of sd-LDL particles were shown to have an approximately 3-fold increase in the risk of developing coronary heart disease compared with individuals with primarily large, buoyant LDL particles [6]. In addition, the sd-LDL-C concentration has been suggested to be a better surrogate marker than the LDL-C concentration for the severity of coronary heart disease [7].

The presence of fatty liver is an independent predictor of coronary heart disease [8-10]. In addition, fatty liver is a manifestation of metabolic syndrome, and is associated with obesity, type 2 diabetes mellitus (T2DM), and hypertriglyceridemia [11]. In patients with T2DM or metabolic syndrome, fatty liver may enhance atherogenesis by increasing levels of sd-LDL particles [12,13]. The precise role of fatty liver in the pathogenesis of sd-LDL, however, is still unclear. In the present study, we

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performed a cross-sectional analysis of a cohort of 476 men to examine potential associations between the fatty liver and sd-LDL levels.

## Materials and methods

Four hundred seventy-six Japanese men who received regular health checkup in 2004 or 2005 participated in the study. Subjects were not taking medication for dyslipidemia and/or diabetes mellitus, and had serum triglyceride (TG) levels less than 400 mg/dl. This study was conducted at Kagoshima Kouseiren Medical Health Care Center, and was approved by the ethics committee of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare.

Fatty liver diagnoses were made using ultrasonography (SSA-250A and SSA-700A, Toshiba, Ibaraki, Japan; Logic 400, GE Yokogawa, Tokyo, Japan) based on findings of a bright liver (increased echogenicity) with liver – kidney contrast (increased echogenicity of the liver compared with the right kidney). Body mass index (BMI) was calculated using the standard equation: body weight (kg)/height$^2$ (m$^2$). Obesity was defined as BMI values ≥ 25 kg/m$^2$. Venous blood samples were obtained from all subjects before 9:00 am after an overnight fast and analyzed immediately. Alanine aminotransferase (ALT) and γ-glutamyl transpeptidase (γ-GTP) activities and serum concentrations of total cholesterol (TC), TG, and glucose were measured using standard laboratory procedures. HDL-C levels were determined using detergents and direct homogeneous assays of serum samples (Daiichi Chemicals, Takaoka, Japan). sd-LDL-C levels were determined using a previously described method with minor modifications [14,15] and a commercially available assay kit (sd-LDL SEIKEN, Denka Seiken Co., Tokyo, Japan). Subjects with TG levels ≥ 400 mg/dl were excluded from this study. LDL-C concentrations were calculated using the Friedewald formula: LDL-C (mg/dl) = TC (mg/dl) – HDL-C (mg/dl) – TG (mg/dl)/5. Serological testing for hepatitis B surface antigen (HBs Ag) and hepatitis C virus antibodies (HCV Ab) was performed using an enzyme immunoassay and enzyme-linked immunosorbent assay, respectively.

Patients were defined as hypertension based on systolic blood pressure ≥ 130 mmHg, diastolic blood pressure ≥ 85 mmHg, or if they were taking medication for hypertension. Hyperglycemia or diabetes was identified based on fasting blood glucose levels ≥ 110 mg/dl or ≥ 126 mg/dl, respectively. Hypertriglyceridemia and elevated levels of LDL-C were defined as TG levels ≥ 150 mg/dl and LDL-C levels ≥ 140 mg/dl, respectively [16]. A common questionnaire was used by a public health nurse to assess each subject's history of alcohol intake and smoking status. Current alcohol consumption was defined as daily alcohol intake of ≥ 20 g/day.

### Statistical analysis

Continuous variables were analyzed using *t*-tests or analysis of variance (ANOVA), and categorical variables were examined using chi-square tests. Multiple comparisons among the differences were examined using Dunnett's or Ryan's method. Maximum likelihood of odds ratios for fatty liver risk and 95% confidence intervals (95% CIs) were calculated using logistic regression models. All presented $P$ values are two-sided. $P$ values $< 0.05$ were considered significant. Statistical analyses were performed using JMP version 7 (SAS Corp, Cary, NC, USA) and R version 2.13.0.

## Results

### Characteristics of the subjects, clinical parameters, and relationships to the presence of fatty liver

Subject demographics, clinical parameters, and associations with fatty liver are summarized in Table 1. Among 476 subjects, 190 (39.9%) were diagnosed with fatty liver (Table 1). The mean concentration [95% CI] of sd-LDL-C among all subjects was 44.6 mg/dl [43.1 – 46.2]. The prevalences of hyperglycemia and diabetes were 29.0% and 8.0%, respectively.

Of note, the lipid profiles of subjects with fatty liver were typically atherogenic (Table 1); the concentrations of TC, TG, LDL-C, sd-LDL-C, and apolipoprotein B were significantly higher in the group with fatty liver than in the subjects without fatty liver. In addition, the concentration of HDL-C was significantly lower in the fatty liver group than in the subjects without fatty liver.

Subjects with fatty liver were significantly younger than those without fatty liver (Table 1). BMI values, the prevalence of obesity, serum glucose concentrations, and the prevalence of hyperglycemia were significantly higher in the fatty liver group compared to the subjects without fatty liver. No significant differences were noted between the groups in the prevalence of hypertension, current alcohol consumption, and smoking status.

### Contributions of hypertriglyceridemia and elevated levels of LDL-C to serum sd-LDL-C concentrations and the prevalence of fatty liver

Features of four groups categorized based on the presence of elevated LDL-C levels (≥ 140 mg/dl) and/or hypertriglyceridemia (≥ 150 mg/dl) are shown in Table 2. sd-LDL-C concentrations were higher in the group with only elevated levels of LDL-C (group B; $P < 0.001$) and the group with only hypertriglyceridemia (group C; $P < 0.001$) compared with the control group (group A). Additionally, sd-LDL-C concentrations in the group with elevated levels of LDL-C and hypertriglyceridemia were the highest among the groups (Group D; $P < 0.001$ compared with the control group). Compared with the control group, the groups with hypertriglyceridemia had

**Table 1 Characteristics of the study subjects**

| | Overall | Fatty liver (–) | Fatty liver (+) | *P* values |
|---|---|---|---|---|
| | (476) | (286) | (190) | Fatty liver (–) vs.(+) |
| Age (years) | 55.9 [55.1–56.8] | 57.8 [56.5–58.9] | 53.2 [51.9–54.4] | < 0.001 |
| BMI (kg/m$^2$) | 24.2 [23.9–24.5] | 23.1 [22.7–23.4] | 25.9 [25.5–26.3] | < 0.001 |
| Obesity (% of subjects) | 37.0 | 23.8 | 56.8 | < 0.001 |
| Blood glucose (g/dl) | 106.8 [105.2–108.4] | 104.5 [102.4–106.5] | 110.2 [107.7–112.8] | < 0.001 |
| Hyperglycemia (% of subjects) | 29.0 | 24.5 | 35.8 | 0.008 |
| TG (mg/dl) | 132.5 [125.9–139.2] | 112.6 [104.5–120.7] | 162.6 [152.7–172.5] | <0.001 |
| HDL-C (mg/dl) | 55.7 [54.5–56.9] | 58.0 [56.4–59.6] | 52.2 [50.5–53.9] | < 0.001 |
| LDL-C$^{\$}$ (mg/dl) | 130.2 [127.6–132.9] | 127.4 [124.1–130.7] | 134.5 [130.1–138.8] | 0.010 |
| TC (mg/dl) | 212.4 [209.5–215.3] | 208.0 [204.3–211.7] | 219.1 [214.4–223.9] | < 0.001 |
| sd-LDL-C (mg/dl) | 44.6 [43.1–46.2] | 40.8 [38.9-42.8] | 50.5 [48.0-53.0] | < 0.001 |
| Apolipoprotein B (mg/dl) | 97.8 [96.–99.6] | 93.2 [91.0–95.4] | 104.6 [101.9–107.3] | < 0.001 |
| ALT (IU/L) | 28.1 [26.4–29.7] | 22.7 [20.8–24.6] | 36.1 [33.7–38.5] | < 0.001 |
| γ-GTP (IU/L) | 45.4 [41.2–49.5] | 36.9 [31.7–42.1] | 58.0 [51.6–64.4] | < 0.001 |
| Hypertension (% of subjects) | 54.8 | 52.5 | 58.4 | 0.200 |
| Current alcohol consumption (%) | 37.8 | 40.2 | 34.2 | 0.186 |
| Smoking status (% of subjects) (never/former/current) | 31.5/39.9/28.6 | 31.8/39.5/28.7 | 31.1/40.5/28.4 | 0.974 |

Data are expressed as means [95% CIs] or percentages.
$^{\$}$, LDL-C levels were calculated using the Friedwald formula.
Continuous variables were compared by *t*-test and categorical variables were compared by Chi-square test.

higher proportions of sd-LDL-C (group C and group D; both $P < 0.001$), whereas group B did not show a significant difference ($P = 0.766$).

No significant differences were observed between groups A and B in BMI values ($P = 0.719$), the prevalence of obesity, serum glucose levels ($P = 0.838$), and the prevalence of impaired fasting glucose levels. The prevalence of fatty liver in group B ($P = 0.001$), however, was significantly higher than in group A. No significant difference between groups A and B was noted for the concentration of HDL-C ($P = 0.235$). BMI values were significantly higher in group C than in group A ($P = 0.001$). The prevalence of obesity was higher in group C than in group A, although the difference was not significant (Ryan's method).

The mean ages ($P = 0.024$), blood glucose levels ($P = 0.021$), and rates of hyperglycemia ($P = 0.006$) significantly differed in groups A and C. Furthermore, the prevalence of fatty liver was significantly higher in group C than in group A ($P < 0.001$). The concetration of HDL-C was lower in group C than in group A ($P < 0.001$).

Compared with group A, group D had higher BMI values ($P < 0.001$), blood glucose concentrations ($P < 0.001$), and rates of obesity ($P = 0.003$), hyperglycemia ($P = 0.003$), and fatty liver ($P < 0.001$), whereas the mean age ($P < 0.001$) and HDL-C concentration ($P < 0.001$) were lower.

### Independent association between fatty liver and sd-LDL-C levels

The subjects were classified into four groups based on serum sd-LDL-C concentrations: ≤ 21.1 mg/dl, 21.2–42.7 mg/dl, 42.8–55.7 mg/dl, and ≥ 55.8 mg/dl. Characteristics of the groups are summarized in Table 3. Among these groups, the concentrations of TG, LDL-C, TC, and apolipoprotein B increased significantly and the HDL-C concentration decreased significantly as sd-LDL-C levels increased. BMI values and the rates of fatty liver and hyperglycemia increased significantly with increasing sd-LDL-C levels. No significant association was detected between sd-LDL-C levels and the prevalence of hypertension, current alcohol consumption, or current smoking.

As shown in Table 4, logistic regression analysis revealed an independent association between sd-LDL-C levels and the presence of fatty liver using BMI values, the presence of hyperglycemia, and other potential confounders as variables. This association remained when the analysis was performed only with subjects who did not have elevated levels of LDL-C (subjects in groups A and C from Table 2) or with those who did not have hypertriglyceridemia (subjects in groups A and B from Table 2).

## Discussion

This study provides important information about the relationship between fatty liver and LDL particle size. We

**Table 2 Comparison of groups categorized based on the presence of elevated LDL-C levels and hypertriglyceridemia**

| Elevated LDL-C levels/ hypertriglyceridemia | Group A (−)/(−) (n = 216) | Group B (+)/(−) (n = 120) | Group C (−)/(+) (n = 95) | Group D (+)/(+) (n = 45) | *P* value |
|---|---|---|---|---|---|
| Lipid profiles | | | | | |
| TG (mg/dl) | 91.1 [85.1–96.7] | 98.1 [90.5–105.6] | 229.3 [220.8–237.8] | 219.0 [206.7–231.4] | < 0.001 |
| HDL-C (mg/dl) | 59.3 [57.6–61.1] | 56.8 [54.4–59.1] | 49.3 [46.7–52.0] | 48.9 [45.1–52.7] | < 0.001 |
| LDL-C (mg/dl) | 114.2 [111.8–116.7] | 162.5 [159.2–165.8] | 112.1 [108.4–115.8] | 159.1 [153.8–164.5] | < 0.001 |
| Sd-LDL-C (mg/dl) | 33.8 [31.9–35.6] | 49.6 [47.2–52.1] | 51.9 [49.2–54.7] | 68.5 [64.5–72.5] | < 0.001 |
| Sd-LDL-C/LDL-C | 0.30 [0.28–0.31] | 0.30 [0.29–0.32] | 0.47 [0.45–0.48] | 0.43 [0.40–0.46] | < 0.001 |
| TC (mg/dl) | 191.8 [188.7–194.8] | 238.9 [234.8–243.1] | 207.3 [202.7–212.0] | 251.8 [245.1–258.6] | < 0.001 |
| Apolipoprotein B (mg/dl) | 82.8 [75.5–80.4] | 112.6 [110.3–114.9] | 99.4 [96.8–102.0] | 126.3 [122.5–130.1] | < 0.001 |
| Demographics, clinical characteristics, and laboratory data | | | | | |
| Age (years) | 57.4 [56.2–58.7] | 56.1 [54.4–57.8] | 54.3 [52.4–56.3] | 51.5 [48.7–54.3] | < 0.001 |
| BMI (kg/m$^2$) | 23.6 [23.2–24.0] | 24.0 [23.4–24.5] | 25.1 [24.5–25.7] | 25.7 [24.8–26.6] | < 0.001 |
| Obesity (% of subjects) | 31.0 | 30.8 | 46.3 | 62.2 | < 0.001 |
| Glucose (mg/dl) | 103.8 [101.4–106.1] | 105.2 [102.1–108.4] | 113.5 [110.0–117.1] | 111.5 [106.4–116.7] | < 0.001 |
| Hyperglycemia | | | | | |
| (% of subjects) | 22.7 | 27.5 | 37.9 | 44.4 | 0.004 |
| ALT (IU/L) | 24.3 [22.0–26.6] | 27.4 [24.3–30.5] | 34.4 [30.9–37.9] | 34.6 [29.5–39.6] | < 0.001 |
| γ-GTP (IU/L) | 35.3 [29.4–41.2] | 38.6 [30.7–46.5] | 70.0 [61.2–78.9] | 59.6 [46.8–72.5] | < 0.001 |
| Fatty liver (% of subjects) | 25.9 | 43.3 | 61.1 | 53.3 | < 0.001 |
| Hypertension | | | | | |
| (% of subjects) | 53.7 | 54.2 | 57.9 | 55.6 | 0.919 |
| Current alcohol consumption | | | | | |
| (% of subjects) | 38.4 | 54.2 | 46.3 | 33.3 | 0.153 |
| Current smokers | | | | | |
| (% of subjects) | 28.7 | 30.0 | 28.4 | 24.4 | 0.919 |

Data are expressed as means [95% CIs] or percentages.
[$], LDL-C levels were calculated using the Friedwald formula.
Continuous variables were compared by ANOVA and categorical variables were compared by Chi-square tests.

revealed an independent association between the presence of fatty liver and serum sd-LDL-C levels, including after we adjusted for such potential confounders as BMI and impaired fasting glucose levels. It may be problematic to discuss visceral obesity and insulin resistance only based on BMI and impaired fasting glucose levels, respectively. Toledo and colleagues, however, showed a relationship between fatty liver and sd-LDL size in patients with T2DM [12]. Sugeno and colleagues suggested that fatty liver synergistically interacts with metabolic syndrome to affect sd-LDL-C levels [13]. Based on these studies and the data present here, fatty liver appears to affect LDL particle size, an effect that may be independent of visceral obesity and systemic insulin resistance. Thus, treating fatty liver may decrease atherogenesis in the patients with metabolic syndrome or T2DM by reducing sd-LDL-C levels.

The composition of TG and cholesterol esters in LDL particles is modified through interactions with TG-rich lipoproteins and cholesterol ester transfer protein (CETP); the molecules are sequentially hydrolyzed by lipoprotein lipase, resulting in the generation of sd-LDL particles [1]. Therefore, elevated LDL-C levels and hypertriglyceridemia seem to be the causative dyslipidemia of sd-LDL particles formation. Tokuno and colleagues reported that statin and fibrate decrease sd-LDL-C concentrations in patients with T2DM via different mechanisms: the former does not affect the sd-LDL-C/LDL-C ratio, whereas the latter reduces this ratio [17]. In the present study, sd-LDL-C concentrations were significantly elevated in subjects with elevated LDL-C levels and normal TG levels, although the sd-LDL-C/LDL-C ratio did not increase. In contrast, sd-LDL-C concentrations and the sd-LDL-C/LDL-C ratio were elevated in subjects with normal LDL-C levels and hypertriglyceridemia (Table 2). Notably, fatty liver was significantly more common in both of these groups. In addition, as shown in Table 4, an independent association between fatty liver

**Table 3 Characteristics of groups categorized based on serum sd-LDL-C concentrations**

| Characteristics | Quartiles of serum sd-LDL-C concentrations (mg/dl) | | | | |
|---|---|---|---|---|---|
| | ≤ 21.1 (n = 119) | 21.2 - 42.7 (n = 119) | 42.8 - 55.7 (n = 118) | 55.8 (n = 120) | *P* value |
| Lipid profiles | | | | | |
| TG (mg/dl) | 83.8 [72.6–95.1] | 111.8 [100.5–123.1] | 145.3 [134.0–156.7] | 188.7 [177.5–200.0] | < 0.001 |
| HDL-C (mg/dl) | 63.5 [61.2–65.8] | 57.6 [55.3–59.8] | 52.7 [50.4–55.0] | 49.0 [46.7–51.3] | < 0.001 |
| LDL-C[$] (mg/dl) | 110.3 [105.7–115.0] | 125.9 [121.2–130.6] | 135.9 [131.2–140.5] | 148.6 [144.0–153.3] | < 0.001 |
| TC (mg/dl) | 190.6 [185.6–195.7] | 205.8 [200.8–210.9] | 217.7 [212.6–222.8] | 235.4 [230.4–240.4] | < 0.001 |
| Apolipoprotein B (mg/dl) | 78.0 [75.5–80.4] | 91.9 [89.4–94.4] | 103.5 [101.0–105.9] | 117.6 [115.1–120] | < 0.001 |
| Demographics, clinical characteristics, and laboratory data | | | | | |
| Age (years) | 57.9 [56.1–59.6] | 56.8 [50.1–58.6] | 55.3 [53.6–57.1] | 53.8 [52.0–55.5] | 0.007 |
| BMI (kg/m$^2$) | 23.4 [22.8–23.9] | 24.0 [23.4–24.5] | 24.5 [24.0–25.1] | 25.0 [24.4–25.5] | < 0.001 |
| Obesity (% of subjects) | 30.3 | 30.3 | 39.8 | 47.5 | 0.017 |
| Glucose (mg/dl) | 105.0 [101.8–108.2] | 105.6 [103.0–106.2] | 10.6.2 [103.8–108.6] | 110.4 [105.9–115.0] | 0.080 |
| Hyperglycemia | | | | | |
| (% of subjects) | 20.2 | 28.6 | 30.5 | 36.7 | 0.045 |
| ALT (IU/L) | 22.4 [19.3–25.5] | 26.5 [23.3–29.6] | 29.1 [26.0–32.3] | 34.3 [31.2–37.4] | < 0.001 |
| γ-GTP (IU/L) | 31.9 [23.8–39.9] | 40.5 [32.4–48.6] | 47.0 [38.9–55.1] | 62.0 [53.9–70.0] | < 0.001 |
| Fatty liver (% of subjects) | 23.5 | 34.5 | 44.0 | 57.5 | < 0.001 |
| Hypertension | | | | | |
| (% of subjects) | 52.2 | 52.1 | 53.4 | 61.7 | 0.380 |
| Current drinking | | | | | |
| (% of subjects) | 39.5 | 39.5 | 39.8 | 32.5 | 0.587 |
| Current smoking | | | | | |
| (% of subjects) | 23.5 | 32.8 | 25.4 | 32.5 | 0.262 |

Data are expressed as means [95% corresponding intervals] or percentages.
[$], LDL-C levels were calculated using the Friedwald formula.
Continuous variables were compared by ANOVA and categorical variables were compared by Chi-square tests.

and sd-LDL-C levels was observed when multivariable analysis was performed using only subjects without elevated LDL-C levels (subjects in groups A and C from Table 2) or those without hypertriglyceridemia (subjects in groups A and B from Table 2). Therefore, fatty liver appears to independently affect LDL particle size owing, at least in part, to impaired hepatic metabolism of TG and LDL-C. The concentration of sd-LDL-C were highest in the group with elevated LDL-C levels and hypertriglyceridemia (group D from Table 2), suggesting that the effects of impaired metabolism of TG and LDL-C may be additive.

Visceral obesity and insulin resistance have been recognized as major causes of increased levels of sd-LDL particles, because these factors are major contributors to postprandial hypertriglyceridemia; one underlying

**Table 4 Association between fatty liver and sd-LDL-C levels**

| Characteristics | Quartiles of serum sd-LDL-C concentrations (mg/dl) | | | | |
|---|---|---|---|---|---|
| | ≤ 21.1 | 21.2 - 42.7 | 42.8 - 55.7 | ≥ 55.8 | *P* value for trend |
| All subjects | 1 (referent) | 1.59 [0.85–3.20] | 1.96 [1.05–3.69] | 2.48 [1.32–4.70] | 0.001*** |
| Subsets of subjects | | | | | |
| HBs-Ag (–), HCV-Ab(–)[#] | 1 (referent) | 1.83 [0.85–4.05] | 2.50 [1.18–5.43] | 3.60 [1.73–7.77] | 0.001*** |
| Elevated LDL-C levels (–) [##] | 1 (referent) | 1.60 [0.79–3.26] | 1.95 [0.92–4.16] | 2.89 [1.29–6.62] | 0.009*** |
| Hypertriglyceridemia (–) [###] | 1 (referent) | 1.14 [0.57–2.29] | 1.55 [0.76–3.23] | 2.25 [1.00–5.10] | 0.018*** |

Risk of fatty liver was estimated by logistic regression analysis using sd-LDL-C levels, age, BMI, hypertension, hyperglycemia, current alcohol consumption, and current smoking as covariables. Data are expressed as odds ratios [95% corresponding intervals]. *P* values for trend were obtained using the likelihood ratio test.
[#], Subjects were 385 men who were negative for both HBs-Ag and HCV-Ab. [##], Subjects were 311 men who did not have elevated levels of LDL-C. [###], Subjects were 336 men who did not show hypertriglyceridemia.

mechanism is increased free fatty acid release from adipocytes, which stimulates hepatic TG output. Additionally, if a fatty liver is present, upregulated de novo synthesis of fatty acids may increase hepatic TG production. Donnely et al. reported that approximately 60% of fat that accumulates in the liver and is incorporated into lipoprotein is derived from circulating free fatty acids, and nearly 25% results from de novo lipid synthesis in patients with nonalcoholic fatty liver disease (NAFLD) [18]. In addition to altered TG output, fatty liver has been shown to be associated with increased TG content per very-low-density lipoproteins (VLDL) particle which is defined as large VLDL [12,19]. Large VLDL efficiently promotes modification of LDL particles via CETP. Recent studies revealed that the liver X receptor (LXR)-sterol regulatory element-binding protein (SREBP)-1c pathway governs the size of VLDL particles secreted by the liver [20,21]. It is noteworthy that the LXR-SREBP-1c pathway is a major causative factor of fatty liver, because several genes involved in de novo fatty acid synthesis are expressed in response to upregulated LXR-SREBP-1c signaling [22]. Thus, fatty liver affects VLDL particles quantitatively and qualitatively, resulting in increased sd-LDL formation.

Interestingly, the LXR-SREBP-1c pathway is a key regulator of not only fatty acid metabolism but also cholesterol metabolism [23,24]. Activation of the LXR-SREBP-1c pathway by increased intrahepatic cholesterol levels stimulates cholesterol secretion in VLDL and suppression of LDL uptake. Enhanced de novo synthesis of cholesterol in livers of patients with NAFLD [25] may increase intrahepatic cholesterol concentrations. Excess intake of dietary cholesterol has been observed in patients with NAFLD [26]. We previously showed a positive independent association between the presence of fatty liver and serum cholesterol levels in men [27]. Thus, we believe that fatty liver is independently associated with elevated serum LDL-C levels owing to altered cholesterol metabolism that results in increased sd-LDL-C levels.

The activities of CETP and hepatic lipase may correlate with sd-LDL-C levels [28]. Lipoprotein lipase is responsible for an important step in TG clearance. Prolonged accumulation of TG-rich remnants following meals may also be associated with postprandial dyslipidemia. Thus, further studies are needed to clarify the role of fatty liver in elevated levels of sd-LDL particles. Of note, our study has several limitations. First, the association between fatty liver and sd-LDL-C was examined with multivariate analysis using age, BMI, hypertension, hyperglycemia, current alcohol consumption [29], smoking status [30], and sd-LDL-C levels as covariables. It is possible, however, that additional factors that we did not analyze may have affected the results. For example, we did not enroll women subjects, which may have skewed the results [30]. Second, the diagnosis of fatty liver was made using abdominal ultrasonography, which identifies fatty steatosis. Ultrasonography may not detect a subset of advanced alcohol or nonalcoholic fatty liver diseases—referred to as burnt-out steatohepatitis—which are characterized by less fatty steatosis.

## Conclusion

Fatty liver may affect the hepatic metabolism of TG and/or LDL-C, resulting in increased serum sd-LDL-C levels and accelerated atherogenesis in patients with metabolic syndrome or T2DM. Therefore, fatty liver should be treated, especially if patients present with metabolic syndrome or T2DM. Further studies, however, are needed to develop effective treatment strategies for fatty liver.

### Abbreviations

LDL-C: Low-density lipoprotein cholesterol; sd-LDL-C: Small, dense low-density lipoprotein cholesterol; TG: Triglyceride; T2DM: Type 2 diabetes mellitus; HDL-C: High-density lipoprotein cholesterol; BMI: Body mass index; ALT: Alanine aminotransferase; γ-GTP: γ-glutamyl transpeptidase; TC: Total cholesterol; NAFLD: Nonalcoholic fatty liver disease; VLDL: Very-low-density lipoproteins.

### Competing interests

All authors declare that they have no conflict of interest.

### Authors' contributions

KH, HU and YI researched and analyzed data. HT also participated in the concept and design of the study, interpretation of data and reviewed/edited the manuscript. YH, ET, YH and TK collected the data. KS analyzed data. MO and AI contributed to discussion and wrote the manuscript. All authors read and approved the final version of the manuscript.

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# Liver regeneration after partial hepatectomy in rat is more impaired in a steatotic liver induced by dietary fructose compared to dietary fat

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ABSTRACT

Hepatic steatosis (HS) has a negative effect on liver regeneration, but different pathophysiologies of HS may lead to different outcomes. Male Sprague-Dawley rats were fed a high fructose (66% fructose; H-fruc), high fat (54% fat; H-fat), or control chow diet for 4 weeks. Based on hepatic triglyceride content and oil red O staining, HS developed in the H-fruc group, but was less severe compared to the H-fat group. Hepatic mRNA expression levels of fatty acid synthase and fructokinase were increased and those of carnitine palmitoyltransferase-1 and peroxisome proliferator-activated receptor-α were decreased in the H-fruc group compared to the H-fat group. Liver regeneration after 70% partial hepatectomy (PHx) was evaluated by measuring the increase in postoperative liver mass and PCNA-positive hepatocytes, and was impaired in the H-fruc group compared to the H-fat and control groups on days 3 and 7. Serum levels of tumor necrosis factor-α, interleukin-6 and hepatocyte growth factor did not change significantly after PHx. In contrast, serum TGF-β1 levels were slightly but significantly lower in the control group on day 1 and in the H-fat group on day 3 compared to the level in each group on day 0, and then gradually increased. However, the serum TGF-β1 level did not change after PHx in the H-fruc group. These results indicate that impairment of liver regeneration after PHx in HS is related to the cause, rather than the degree, of steatosis. This difference may result from altered metabolic gene expression profiles and potential dysregulation of TGF-β1 expression.



## 1. Introduction

Nonalcoholic fatty liver disease (NAFLD) is a common clinical problem that results from a variety of metabolic disorders, including obesity, diabetes mellitus, and dyslipidemia. Fatty liver is present in 9.6–29% of autopsy cases [1,2], and moderate hepatic steatosis (30–60% fatty hepatocytes) is found in 6% of autopsies after accidental deaths [2]. The 5-year survival rate of patients with nonalcoholic steatohepatitis (NASH) which is a progressive liver disease in NAFLD, is 75% [3]. Complications of NASH, including cirrhosis and hepatocellular carcinoma (HCC), are expected to increase with the growing epidemic of diabetes and obesity [4]. However, it is unclear which cases of NAFLD are likely to progress to severe fibrosis and HCC.

Obesity has escalated to epidemic proportions worldwide and many causes have been suggested, including dietary components. Excessive caloric intake has been related to high-fat foods and increased portion sizes. The increase in consumption of high-fructose corn syrup (HFCS) has a temporal relationship with the epidemic of obesity, and overconsumption of HFCS in calorically sweetened beverages may have played a role in the epidemic [5]. Excessive fructose and sucrose (which contains 50% fructose) intake is a risk factor for developing NAFLD and may be associated with NASH [6,7]. Therefore, abnormal metabolism may be a pathophysiological feature of hepatic steatosis.

Many growth factors and cytokines such as hepatocytes growth factor (HGF) stimulate liver regeneration. In contrast, transforming growth factor (TGF)-β is a potent inhibitor of hepatocyte proliferation [8] and is involved in growth arrest once the liver has reached an appropriate functional mass [9]. Liver regeneration requires orchestrated functions of these molecules in appropriate amounts and at appropriate locations and times [10]. Hepatic steatosis is associated with an increased incidence of complications and mortality after liver resection or transplantation [11], and these outcomes are associated with a decrease in the ability of the liver to regenerate in NAFLD [12]. However, direct experimental evidence of a relationship between a high fructose or high fat diet and cytokine expression in liver regeneration is lacking.

In this study, we investigated mechanistic differences in liver regeneration in fatty liver and metabolic abnormalities caused by

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dietary fructose or fat. The results may contribute to elucidation of the mechanisms that affect the outcome of NAFLD.

## 2. Materials and methods

### 2.1. Animals and diets

Male Sprague-Daley rats of age 7 weeks were obtained from Kyudo (Kumamoto, Japan). Rats were acclimatized to laboratory conditions for at least 7 days at a constant temperature of 24 °C with a 12-h light-dark cycle, and fed a standard rodent diet (CE-2; Kyudo, Kumamoto, Japan) and water ad libitum. All animal experiments were approved by the Institutional Animal Care and Use Committee guideline of Kagoshima University.

After acclimatization, rats were placed in groups that were fed three different diets for 4 weeks ad libitum: control chow (10% of total calories as fructose, 12% as fat, and 19% as protein; control group), a high fat diet (8% of total calories as fructose, 54% as fat, and 15% as protein; H-fat group), and a high fructose diet (66% of total calories as fructose, 11% as fat, and 19% as protein; H-fruc group). The diets were obtained from Nosan Corporation (Kanagawa, Japan). All diets were prepared as a powder, and diet weights and intake energy were measured every other day.

A 70% partial hepatectomy (PHx) was performed after 4 weeks of intake of each diet. Rats were anesthetized by ether inhalation. After midline laparotomy, the median and left hepatic lobe were pushed out and ligated with 2.0 silk sutures, and resected as described previously [13]. Resected livers were weighed and used for RNA extraction, or thin slices were immersed in 10% formalin and embedded in paraffin to make 4-μm sections for routine staining with hematoxylin and eosin. After PHx, all rats were fed with a standard CE-2 diet and sacrificed on days 1, 3, and 7 after PHx. Blood was collected by vena cava puncture and centrifuged. The resulting serum was stored at −80 °C. The remaining right hepatic lobes were removed and weighed.

### 2.2. Evaluation of insulin sensitivity

Fasting blood glucose (FBG) and serum immunoreactive insulin (IRI) levels were determined by ELISA (Morinaga Institute of Biological Science, Kanagawa, Japan). The homeostasis model assessment-insulin resistance index (HOMA-IR) was calculated from FBG (mg/dl) and IRI (mU/ml) levels as FBG × IRI/405. After 4 weeks diet intake, rats in all groups underwent an intraperitoneal insulin tolerance test (ITT), which was performed after a 4-h fast by intraperitoneal injection of 0.5 U insulin per kg of body weight. Blood was drawn from a tail vein after injection for measurement of plasma glucose concentrations [14].

### 2.3. Assessment of hepatic steatosis

Hepatic steatosis was assessed using hepatic triglyceride levels and oil red O staining. Hepatic lipids were extracted with chloroform-methanol and measured enzymatically using a commercial kit (L-type Wako TGH, Wako Pure Chemical Industries, Osaka, Japan). Oil red O staining was performed to evaluate accumulation of fat droplets in hepatocytes in frozen liver sections. The ratio of the oil red O-stained area to the total area was determined using Image J software (http://rsb.info.nih.gov/ij/index.html).

### 2.4. Assessment of hepatic mRNA levels of genes associated with metabolism of lipids and fructose

The relative levels of specific mRNAs in resected liver on day 0 were assessed by real-time quantitative polymerase chain reaction (RT-qPCR) using Syber Premix Ex Taq (TaKaRa Bio, Shiga, Japan). Total RNA was extracted from each liver using Isogen (Nippon Gene, Tokyo, Japan). Expression levels of target genes were calculated relative to the level of glyceraldehyde-3-phosphate dehydrogenase (GAPDH), which was used as an endogenous control gene to normalize the target gene expression levels. All procedures were performed according to manufacturers' instruction. mRNA levels were determined for carnitine palmitoyltransferase (CPT)-1, peroxisome proliferator-activated receptor (PPAR)-α, AMP-activated protein kinase (AMPK), fatty acid synthase (FAS), fructokinase (FK), and cytochrome P450 2E1 (Cyp2E1). Primer sequences are listed in Table 1. The PCR primers were obtained from TaKaRa Bio Inc.

**Table 1**
Primers used for PCR.

| Gene | Gene Bank Number | Primer sequences | |
|---|---|---|---|
| PPARα | NM_013196.1 | Forward | 5′-GACAAGGCCTCAGGATACCACTATG-3′ |
| | | Reverse | 5′-TTGCAGCTTCGATCACACTTGTC-3′ |
| AMPK | NM_019142.1 | Forward | 5′-GGCTCGCCCAATTATGCTG-3′ |
| | | Reverse | 5′-AGAGTTGGCACGTGGTCATCA-3′ |
| CPT-1 | NM_031559.2 | Forward | 5′-CGCTCATGGTCAACAGCAACTAC-3′ |
| | | Reverse | 5′-TCACGGTCTAATGTGCGACGA-3′ |
| FK | NM_031855.3 | Forward | 5′-CCAGCTGTTCGGCTATGGAGA-3′ |
| | | Reverse | 5′-CACAGCCAACCAGATGCTTCA-3′ |
| FAS | NM_017332.1 | Forward | 5′-CACAGCATTCAGTCCTATCCACAGA-3′ |
| | | Reverse | 5′-TCAGCCCAGGCACAGATGAG-3 |
| Cyp2E1 | NM_031543 | Forward | 5′-CCTACATGGATGCTGTGGTG-3′ |
| | | Reverse | 5′-CTGGAAACTCATGGCTGTCA-3′ |
| GAPDH | NM_017008.3 | Forward | 5′-GGCACAGTCAAGGCTGAGAATG-3′ |
| | | Reverse | 5′-ATGGTGAAGACGCCAGTA-3′ |

### 2.5. Assessment of liver regeneration

The liver regeneration rate (%) was calculated as $100 \times \{C - (A - B)\}/A$, where $A$ is the estimated total liver weight at the time of partial hepatectomy, $B$ is the excised liver weight, and $C$ is the weight of the regenerated liver at the final resection [13]. Liver samples were also stained for proliferating cell nuclear antigen (PCNA). After fixation with formalin and paraffin embedding, tissue was incubated with anti-PCNA (clone PC10; Dako, Tokyo, Japan). The PCNA proliferation index was determined in at least 1000 hepatocytes in each group. Data are expressed as the percentage of PCNA-stained hepatocytes out of the total number of hepatocytes examined.

### 2.6. Assessment of serum markers

Serum level of high sensitivity C-reactive protein (hs-CRP; Rat CRP ELISA, Life Diagnostics, West Chester, PA) was determined using commercially available kits. Serum levels of tumor necrosis factor (TNF)-α, interleukin (IL)-6, TGF-β1 (all R&D Systems, Minneapolis, MN) and HGF (Institute of Immunology, Tokyo, Japan) were measured on days 0, 1, 3 and 7.

### 2.7. Statistical analysis

Statistical comparison among groups was performed using one-way ANOVA or repeated ANOVA and a post-hoc Fisher PLSD test. A Student $t$-test was used for comparison of two groups. $P < 0.05$ was considered statistically significant. Data are presented as the mean ± standard error (SE) or standard deviation (SD).

## 3. Results

### 3.1. Metabolic parameters in rats fed with a high fat or high fructose diet

Total energy intake for 4 weeks and body weight (mean body weight relative to basal body weight) did not differ between the H-fat and H-fruc groups, but were significantly higher in these groups compared to the control group ($P < 0.05$) (Table 2). The serum triglyceride level was higher in the H-fruc group than in the H-fat and control groups. There were no significant differences in serum adiponectin levels among the three groups, but serum leptin levels were significantly higher in the H-fat and H-fruc groups compared to the control group ($P < 0.05$). Serum hs-CRP was significantly higher in the H-fruc group compared to the H-fat group ($P < 0.05$). FBG was significantly higher in the H-fruc group than in the control group ($P < 0.05$). IRI and HOMA-IR showed a tendency to be higher in the H-fruc group (Table 2) and the H-fruc group had significantly more severe insulin resistance based on ITT results ($P < 0.001$) (Fig. 1A).

**Table 2**
Metabolic parameters and serum biochemical markers after 4 weeks of diet intake.

| | Control | H-fat | H-fruc |
|---|---|---|---|
| Body weight (% basal BW) | 140.3 (7.5) | 170.7 (6.3)* | 171.7 (16.4)* |
| Total energy intake (kcal) | 2108.8 (248.4) | 2530.1 (72.0)* | 2419.3 (84.1)* |
| ALT (IU/L) | 33.6 (4.5) | 22.6 (1.8) | 24.8 (5.0) |
| Triglyceride (mg/dl) | 67.2 (23.36) | 62.7 (21.82) | 145.0 (48.19)** |
| Free fatty acids (mEq/L) | 5.63 (3.43) | 8.03 (2.55) | 6.90 (3.25) |
| Fasting blood glucose (mg/dl) | 108.0 (25.0) | 116.8 (18.8) | 144.8 (16.3)* |
| Insulin (ng/ml) | 3.73 (2.37) | 2.76 (1.05) | 4.74 (2.84) |
| HOMA-IR | 1.09 (0.90) | 0.78 (0.21) | 1.77 (1.14) |
| Adiponectin (μg/ml) | 1.71 (0.61) | 2.80 (0.62) | 2.36 (0.51) |
| Leptin (ng/ml) | 0.61 (0.15) | 1.64 (0.62)* | 1.45 (0.50)* |
| Hs-CRP (mg/ml) | 0.13 (0.07) | 0.12 (0.05) | 0.17 (0.06) *** |

Data are shown as mean (standard deviation) after 4-week intake of the three diets. ALT, alanine aminotransferase; HOMA-IR, homeostasis model of assessment for insulin resistance index; and Hs-CRP, high sensitivity C-reactive protein.
* $P < 0.05$ *vs.* Cont.
** $P < 0.01$ *vs.* Cont and H-fat.
*** $P < 0.05$ *vs.* H-fat.

### 3.2. Severity of hepatic steatosis in the H-fat and H-fruc groups

Micro and macrovesicular steatosis were clearly visible on HE staining of livers from H-fat and H-fruc rats (Fig. 1B). The hepatic fat area (%) calculated by oil red O staining and the hepatic triglyceride content were significantly higher in the H-fat group compared to the H-fruc and control groups (Fig. 1C and D).

### 3.3. Hepatic mRNA expression levels in the H-fat and H-fruc groups after PHx

Hepatic mRNA levels of FK and FAS significantly increased in the H-fruc group and significantly decreased in the H-fat group compared to the control group (Fig. 2). In contrast, mRNA levels for CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly lower in the H-fruc group compared to the H-fat group ($P < 0.05$) (Fig. 2).

### 3.4. Liver regeneration after PHx in the H-fat and H-fruc groups

Liver regeneration and the PCNA labeling index at 3 and 7 days after PHx were significantly lower in the H-fruc group than in the

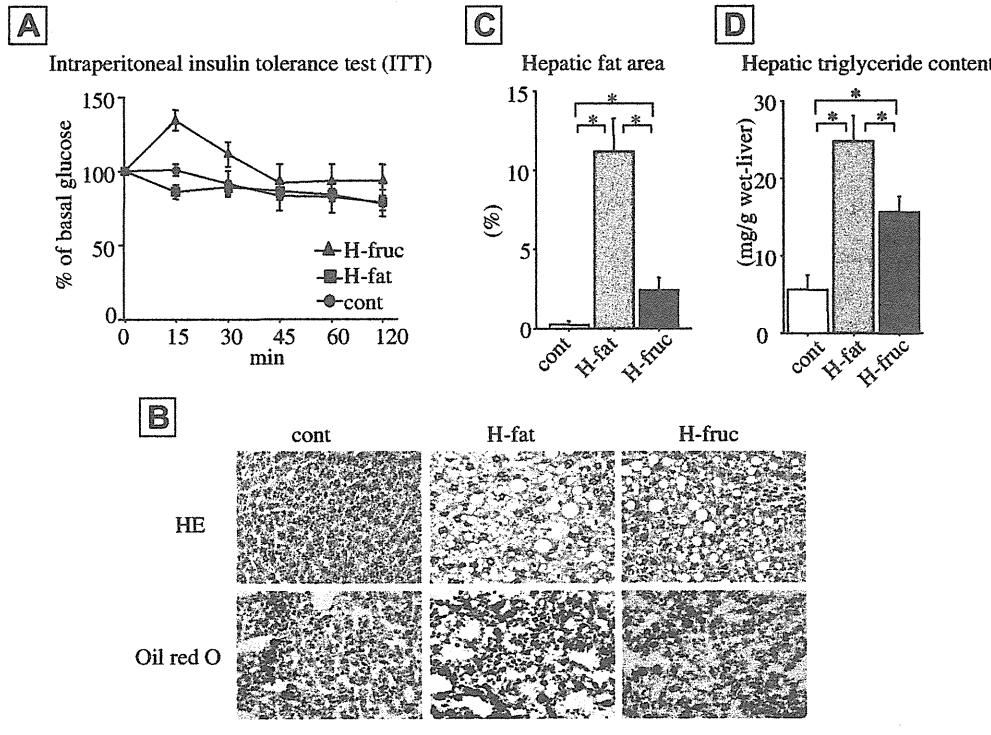


**Fig. 1.** Insulin tolerance test and histological evaluation of fatty liver in rats fed a chow control diet (Cont), a high-fat diet (H-fat) or a high-fructose diet (H-fruc). (A) An intraperitoneal insulin tolerance test (ITT) was performed after 4 weeks intake of each diet. After a 4-h fast, mice were injected intraperitoneally with 0.5 U of insulin per kg body weight. Glucose was measured in blood sampled from a tail vein at 0, 15, 30, 45, 60, and 120 min. H-fruc rats had significantly more severe insulin resistance compared to control and H-fat rats ($n = 5$–6, $P < 0.001$, repeated ANOVA). (B–D) Fatty liver was evaluated by hematoxilin-eosin staining (B), image analysis using oil red O staining (C), and hepatic triglyceride content (D). Fatty infiltration of the liver was most severe in the H-fat group ($n = 6$), and that in the H-fruc group ($n = 6$) was higher than in the control group ($n = 5$). *$P < 0.01$ (one way ANOVA).

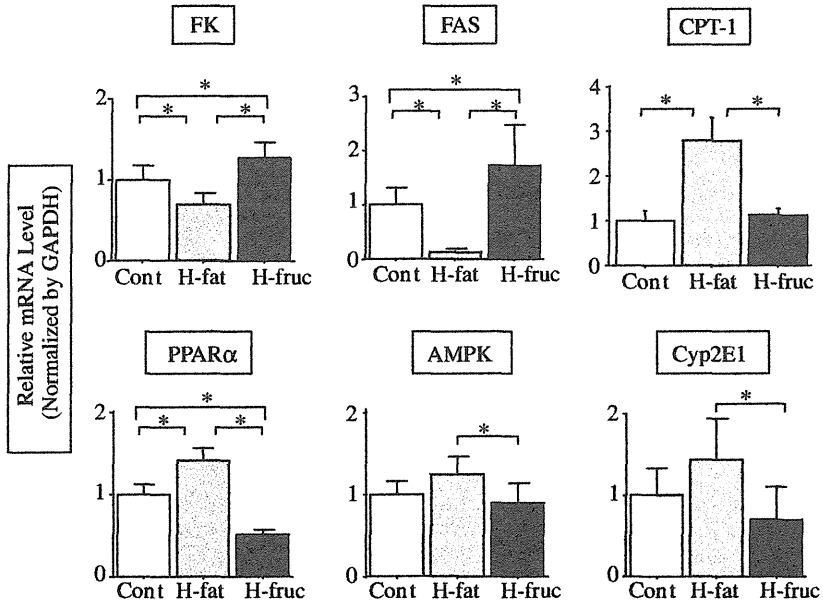


**Fig. 2.** Hepatic mRNA expression of genes involved in metabolism assessed by RT-qPCR (each $n = 5–6$) after 4 weeks of intake of the three diets. Expression levels of CPT-1, PPAR-α, AMPK and Cyp2E1 were significantly suppressed in the H-fruc group compared to the H-fat group. FK and FAS were significantly increased in the H-fruc group compared to the H-fat or control group. In contrast, FAS and FK were significantly decreased in the H-fat group compared to the control group. $^{*}P < 0.05$ (one way ANOVA).

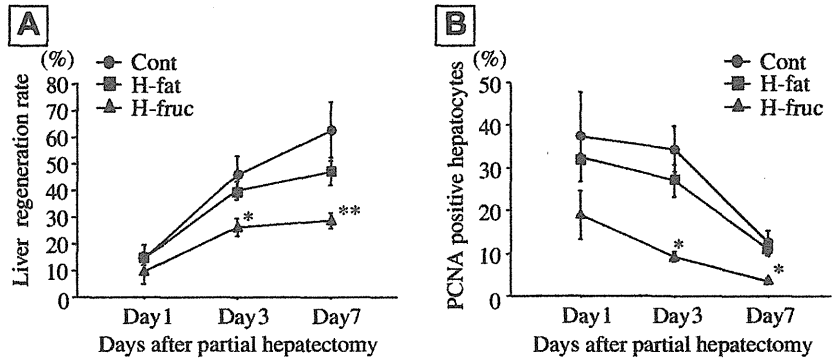


**Fig. 3.** Liver regeneration after 70% partial hepatectomy in rats fed three different diets (each $n = 4–6$). (A) The liver regeneration rate (%) was lowest in the H-fruc group on days 3 and 7. This rate was also lower in the H-fat group compared to the control group, but the difference was not significant. (B) PCNA labeling showed that the mitotic index was significantly lower in the H-fruc group compared to the other two groups. The PCNA proliferation index was determined in at least 1000 hepatocytes. Values are shown as the mean ± standard error ($^{*}P < 0.05$ *vs.* control and H-fat group, $^{**}P < 0.05$ *vs.* control group).

H-fat and control groups (Fig. 3A and B), but only showed a tendency to be lower in the H-fat group compared to the control group.

### *3.5. Serum biochemistry and cytokine levels just before and after PHx*

The serum ALT level in the H-fruc group on day 1 after PHx was significantly higher than that in the H-fat group, but similar to the control group, and there was no difference among the three groups on days 0, 3 and 7 (Fig. 4A). Serum AST, ALP and albumin did not differ significantly before and after PHx. The serum TNF-α level just before PHx showed a tendency to be higher in the H-fruc group, and serum IL-6 and HGF did not differ significantly among the three groups throughout the course of the study (Fig. 4B). In contrast, the serum TGF-β1 level in the control group on day 1 and in the H-fat group on day 3 were significantly lower than the respective levels on day 0, and then gradually increased (Fig. 4B). However, serum TGF-β1 levels were not suppressed after PHx in the H-fruc group.

## 4. Discussion

Studies in animal models and patients with fatty liver have shown impaired liver regeneration that worsens in severe fatty liver or NASH [14–16]. However, the mechanism of impaired liver regeneration in fatty liver is not fully elucidated. In our study, we found that liver regeneration was more impaired in rats given a high fructose diet (H-fruc group) compared to those given a high fat diet (H-fat group), although the severity of hepatic steatosis in the H-fruc group was lower than that in the H-fat group. These results provide the first evidence that liver regeneration is affected by the cause, rather than the severity, of hepatic steatosis. In addition, insulin resistance, altered metabolic gene expression profiles, and abnormal expression of TGF-β1 might be involved in the mechanism of the delay in liver regeneration.

Sucrose, the major component of sugar, is hydrolyzed to glucose and fructose, and increased consumption of sugar and fructose is related to the increase in the overweight population. Fructose in soft drinks and sweetened beverages may be strongly related to

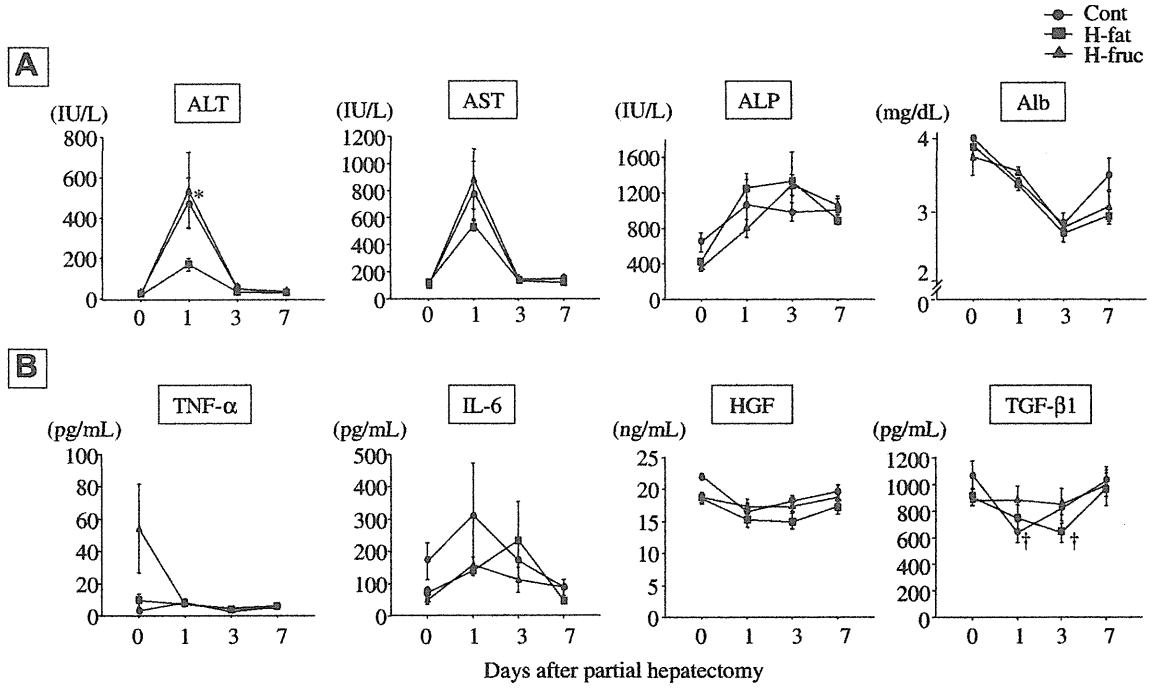


**Fig. 4.** Course of serum chemistry and cytokine levels during liver regeneration after partial hepatectomy (each $n = 4–6$). (A) Hepatocellular damage assessed by serum ALT in the H-fruc and control group on day 1 was higher than that in the H-fat group. However, AST, ALP and albumin did not differ among the three groups during the course of the study. (B) The serum TNF-α level just before partial hepatectomy tended to be higher in the H-fruc group. In contrast, serum TGF-β1 levels in the control group on day 1 and the H-fat group on day 3 were lower than the respective levels on day 0. Serum TGF-β1 levels in the H-fruc group did not change significantly over the course of the study. Values are shown as the mean ± standard error. *$P < 0.05$ *vs.* H-fat group.

obesity [17,18], and excessive intake of sugar and fructose also cause insulin resistance [19]. An overweight status, obesity and insulin resistance are important risk factor for NAFLD, and fructose consumption has been associated with increased hepatic fibrosis in patients with NAFLD [7]. Collectively, these results suggest that sucrose, sugar and fructose are associated with the pathogenesis of NAFLD. Fructose intake is 2- to 3-fold higher in patients with NAFLD compared with gender, age and body mass index-matched controls, and hepatic mRNA expression of FK and FAS is increased in patients with NAFLD [6]. Fructose is absorbed through glucose transporter 5 (GLUT5) in the jejunum. Excess fructose is taken to the liver and phosphorylated by FK for use in gluconeogenesis and lipid production. However, since lipid production from fructose is not regulated, excessive fructose induces production of a large amount of TG [17,18]. The serum TG level and hepatic mRNA levels of FK and FAS in this study were higher in the H-fruc group compared to those in the H-fat or control groups (Table 2 and Fig. 2A). Thus, our experimental model resembles human NAFLD in these respects.

Excessive intake of sugar and fructose may have more adverse effects than excessive intake of a high fat diet. The mortality of rats with hypertension was greater after intake of a high fructose diet compared to a high fat diet [20], and a high sugar diet has been shown to induce cardiac depression and increase mortality [21]. We have also found that liver regeneration was more impaired in the H-fruc group than in the H-fat group for the first time, although these groups did not differ in total energy intake and increase of body weight. Pioglitazone, a drug that improves insulin resistance, has been shown to improve delayed liver regeneration after hepatectomy in KK-Ay mice, which develop insulin resistance [22]. In our study, insulin resistance in the H-fruc group was higher than that in the H-fat group before PHx (Fig. 1A). Thus, the different rates of liver regeneration in the two groups might have been caused by a difference in insulin resistance.

In the liver, ATP is produced as an energy source by carbohydrate metabolism. ATP is also produced by β-oxidation of fatty acids. Fatty acids are oxidized by mitochondria or peroxisomes and are finally transformed to acetyl-CoA. In β-oxidation, ATP is produced from fatty acid. In this process of fatty acid oxidation, CPT-1 is the rate-controlling enzyme, and nuclear receptor transcription factors such as PPAR-α and AMPK regulate expression of CPT-1. In our study, expression of CPT-1 was decreased in rats fed a high fructose diet, and expression of PPARα and AMPK also decreased. The decreased expression of PPAR-α, AMPK and CPT-1 may reduce ATP production [23,24]. On the other hand, expression of FK in the liver increased in H-fruc rats, and this increases consumption of ATP and leads to ATP depletion in the liver [6,25]. Thus, a high fructose diet induced a decrease in ATP production due to a decrease in fatty acid β-oxidation and an increase in ATP consumption due to increased FK expression. These changes produce an overall decrease in ATP, which is essential for liver regeneration, compared to high-fat diet. Delayed liver regeneration has also been shown in PPAR-α null mice [26] and may involve decreased expression of cyclin D1 and c-Myc, which are G1/S phase regulators, and increased expression of IL-1β. Thus, the greater impairment of liver regeneration in the H-fruc group compared to the H-fat group was due to alteration of gene expression profiles.

Liver regeneration after administration of carbon tetrachloride is impaired via abnormal expression of IL-6 and TNF in leptin-deficient ob/ob mice [27]. Picard et al. examined liver regeneration after PHx in methionine-low, choline-deficient diet-fed rats and Zucker fatty rats, and suggested that abnormal function of the leptin receptor might be related to impaired liver regeneration, rather than fatty liver itself [28]. We did not examine leptin receptor expression in this study, but the serum leptin and adiponectin levels did not differ between the H-fruc and H-fat groups. This suggests that changes in the leptin pathway were

not involved in the different rates of liver regeneration in the two groups.

Serum TGF-β1 levels were slightly but significantly lower on day 1 in control rats and on day 3 in H-fat rats, compared to the respective levels just before PHx. However, serum TGF-β1 in the H-fruc group did not change in liver regeneration from day 0 to day 7 (Fig. 4). TGF-β1 is a potent inhibitor of cell proliferation. In a small-for-size liver graft model, hepatic TGF-β1 increased slightly after transplantation of full-size grafts and then decreased rapidly. TGF-β1 progressively increased after transplantation of quarter-size grafts and hepatocyte proliferation was markedly inhibited [10]. TGF-β1 enhanced by LPS also plays an important role in the mechanism of hepatic failure due to infection after hepatectomy, especially in inhibition of liver regeneration [29]. In contrast, inhibition of TGF-β/Smad signaling improves regeneration of small-for-size rat liver grafts, and a single dose of anti-TGF-β1 monoclonal antibody enhances liver regeneration after partial hepatectomy in biliary-obstructed rats [30]. It is possible that dysregulation of TGF-β expression was one of the causes of delayed liver regeneration in the H-fruc rats. However, there were only small differences in serum TGF-β1 levels in control and H-fat rats after PHx. In addition, we did not examine the TGF-β/Smad signaling pathway in the liver of these rats. Therefore, a further study is needed to elucidate the association of dysregulation of TGF-β expression and delayed liver regeneration in the H-fruc rat.

In conclusion, fatty liver was less severe in the H-fruc group than in the H-fat group, but liver regeneration was more impaired in the H-fruc group. These results suggest that impaired liver regeneration in fatty liver is related to the cause, but not necessarily to the degree, of hepatic steatosis. Dysregulation of genes associated with metabolism or ATP production and potential dysregulation of TGF-β1 expression may contribute to impairment of liver regeneration after PHx in hepatic steatosis induced by a fructose diet.

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ORIGINAL ARTICLE—LIVER, PANCREAS, AND BILIARY TRACT

# Impact of cigarette smoking on onset of nonalcoholic fatty liver disease over a 10-year period

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**Abstract**
*Background* Metabolic syndrome, which includes obesity, hyperglycemia, dyslipidemia, and hypertension, is a major risk factor for the development of nonalcoholic fatty liver disease (NAFLD). Cigarette smoking is a well-known risk factor for metabolic syndrome, but the epidemiological impact of cigarette smoking on development of NAFLD is unclear.
*Methods* In this retrospective study, 2,029 subjects underwent a complete medical health checkup in 1998 and again in 2008. Those who were positive for hepatitis B surface antigen or hepatitis C virus antibody, or had an alcohol intake of >20 g/day as assessed by questionnaire, were excluded. Fatty liver was diagnosed by abdominal ultrasonography. Independent risk factors associated with the development of NAFLD were determined by multiple logistic regression analysis. Smoking status was expressed using the Brinkman index (BI), which was calculated as the number of cigarettes smoked per day multiplied by the number of years of smoking.
*Results* Of 1,560 subjects without NAFLD in 1998, 266 (17.1%) were newly diagnosed with NAFLD in 2008. Multiple logistic analysis identified age [adjusted odds ratio (AOR) 0.95, 95% confidence interval (95% CI) 0.94–0.97], male sex (AOR 1.46, 95% CI 1.01–2.10), body mass index ≥25 (AOR 3.08, 95% CI 2.20–4.32), dyslipidemia (AOR 1.79, 95% CI 1.25–2.58) and cigarette smoking (AOR 1.91, 95% CI 1.34–2.72) as risk factors associated with the development of NAFLD. Smoking status at baseline was also associated with the development of NAFLD (BI 1–399: AOR 1.77, 95% CI 1.02–3.07, BI ≥400: AOR 2.04, 95% CI 1.37–3.03).
*Conclusion* Cigarette smoking is an independent risk factor for onset of NAFLD.

**Keywords** NAFLD · Risk factor · Brinkman index · Body mass index · Metabolic syndrome

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## Introduction

Nonalcoholic fatty liver disease (NAFLD) is a liver disorder characterized by fatty changes of the liver with no apparent history of habitual alcohol intake. NAFLD was initially considered to be a reversible chronic liver disease with a favorable prognosis. However, some NAFLD patients show evidence of nonalcoholic steatohepatitis (NASH), which may progress to hepatic cirrhosis and hepatocellular carcinoma, with a resultant unfavorable prognosis [1, 2]. There are also racial differences in the prevalence of NAFLD. In Japan, the prevalence is reported to be 9–30% [3]. The prevalence of visceral fat-type obesity is high in Asian populations, and this may lead to insulin resistance and an increased incidence of NAFLD, even though the body mass index (BMI) of Asians is

generally lower than that of African-Americans and Caucasians [4–6]. A high rate of NAFLD also occurs concomitantly with obesity, hyperglycemia, dyslipidemia, and hypertension (collectively referred to as metabolic syndrome) [3, 4, 7–9], but few large-scale long-term studies of the risk factors involved in the development of NAFLD have been reported.

Cigarettes contain more than 4,000 toxic chemicals, including tar, nicotine, and carbon monoxide. Cigarette smoking is a risk factor for the prevalence of and mortality from malignant cancers such as lung and esophageal cancers, lung diseases such as chronic obstructive pulmonary disease (COPD), and circulatory diseases [10–12]. An association of cigarette smoking with risk factors for NAFLD, such as insulin resistance, diabetes, and dyslipidemia, has also been reported [13–18]. However, a large-scale long-term study of the association between cigarette smoking and NAFLD has not been performed. Therefore, in this study, we investigated the factors involved in the development and cure of NAFLD in a follow-up study of a 10-year interval, and examined the association of smoking with the development of NAFLD.

## Subjects and methods

### Study design

We designed a retrospective follow-up study of a 10-year interval to investigate the effects of cigarette smoking on the development or cure of NAFLD. A total of 3,365 subjects underwent a complete medical health checkup including abdominal ultrasonography at the Kagoshima Kouseiren Medical Healthcare Center in both 1998 and 2008. Subjects positive for hepatitis B virus surface antigen (HBsAg) and hepatitis C virus antibody (HCV Ab) and those who did not undergo virus marker measurements were excluded. Alcohol intake was investigated by questionnaire, and the ethanol equivalent of alcohol consumption per day was calculated from the frequency of alcohol intake per month. Subjects who drank >20 g/day of ethanol were excluded from the study.

The diagnosis of fatty liver was based on the results of abdominal ultrasonography, which was performed by trained technicians. Fatty liver was diagnosed when hepatorenal echo contrast and liver brightness were observed [19, 20]. The diagnosis of fatty liver was subsequently confirmed by a specialist physician independently without reference to other data.

Although abdominal obesity (abdominal circumference >85 cm for men and >90 cm for women) is a necessary variable according to the Japanese criteria for diagnosing metabolic syndrome [21], waist measurements were not available for all the subjects in this study. In addition, a BMI of $\geq$25 has been proposed as a cutoff for the diagnosis of obesity in Asian people [19–22]. Therefore, we defined obesity as a BMI $\geq$25 and included it as one of the metabolic syndrome risk factors in this study. BMI was calculated by dividing body weight (kg) by the square of height ($m^2$). Patients with hypertension were defined as those with a systolic blood pressure of $\geq$130 mmHg, those with a diastolic blood pressure of $\geq$85 mmHg, or those who were undergoing medical treatment for hypertension in 1998. Patients with dyslipidemia were defined as those with triglycerides of $\geq$150 mg/dL, those with HDL <40 IU/L, or those who were undergoing medical treatment of dyslipidemia in 1998. Patients with dysglycemia, including diabetes mellitus, were defined as those who had a fasting plasma glucose of $\geq$110 mg/dL or who were under medical treatment for diabetes in 1998. Thus, hypertension, dyslipidemia and dysglycemia were defined as risk factors for metabolic syndrome in this study according to the Japanese criteria for diagnosing this disorder [20, 21].

Cigarette smoking was investigated by questionnaire, and the Brinkman index (BI) was calculated as the number of cigarettes smoked per day multiplied by the number of years that the subjects had smoked. Subjects who stopped smoking before 1998 (former smokers) or started smoking after 1998 (new smokers) were classified as nonsmokers in 1998, and those who stopped smoking after 1998 and before 2008 (new quitters) were classified as smokers in 1998. We performed further subanalysis using two groups (subjects who continued to smoke between 1998 and 2008, and those who did not smoke at all during this time) or three groups (the previous two groups in addition to new quitters). For alcohol consumption per day, the subjects were divided into 2 groups: those who did not drink alcohol (consumption 0 g/day) and light drinkers (mean consumption $\leq$20 g/day).

The study was approved by the ethics committees of the Kagoshima Prefectural Federation of Agricultural Cooperatives for Health and Welfare and the Kagoshima University Graduate School of Medical and Dental Sciences.

### Statistical analysis

All analyses were performed using SPSS v.18 (SPSS, Inc., Chicago, IL, USA), with the significance level set at <5%. Continuous variables are shown as mean ± standard deviations (SD). Between-group comparison was performed by unpaired *t* test and Fisher's exact test. Potential factors involved in the development or cure of NAFLD were analyzed by logistic regression analysis. Unadjusted and adjusted odds ratios (OR) and 95% confidence interval (95% CI) were calculated.

**Table 1** Baseline characteristics of subjects enrolled in 1998

| Characteristic | NAFLD ($n$ = 469) | Non-NAFLD ($n$ = 1,560) | $P$ value[a] |
|---|---|---|---|
| Mean age, year ± SD | 49.2 ± 8.9 | 51.1 ± 9.3 | <0.001 |
| Men, $n$ (%) | 342 (72.9) | 772 (49.5) | <0.001 |
| BMI ≥25 kg/m$^2$, $n$ (%) | 276 (58.8) | 251 (16.1) | <0.001 |
| Hypertension, $n$ (%)[b] | 198 (42.2) | 422 (27.1) | <0.001 |
| Dyslipidemia, $n$ (%)[b] | 202 (43.1) | 216 (13.8) | <0.001 |
| Dysglycemia, $n$ (%)[b] | 185 (39.4) | 322 (20.6) | <0.001 |
| Current smoker, $n$ (%) | 130 (27.8) | 315 (20.2) | 0.001 |
| Light alcohol drinker, $n$ (%)[b] | 310 (66.1) | 905 (58.0) | <0.01 |

*NAFLD* nonalcoholic fatty liver disease, *BMI* body mass index

[a] Calculated by Fisher's exact test for categorical variables or unpaired $t$ test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

## Results

### Subjects' baseline characteristics in 1998

Of the initial 3,365 subjects, 76 were positive for HBsAg, 90 were positive for HCV Ab, and 2 were positive for both. Four hundred thirty-nine subjects were not tested for HBsAg or HCV Ab. In addition, 729 subjects drank >20 g/day of ethanol. On the basis of these data, 2,029 subjects were eligible for the study.

In 1998, 469 subjects (342 men and 127 women) and 1,560 subjects (772 men and 788 women) were included in the NAFLD and non-NAFLD groups, respectively. There was a significantly higher number of men in the NAFLD group, and the mean age in the NAFLD group was significantly lower than that in the non-NAFLD group (Table 1). The frequencies of obesity (BMI ≥25), hypertension, dyslipidemia, dysglycemia including diabetes mellitus, current cigarette smoking, and light alcohol drinkers were significantly higher in the NAFLD group compared to the non-NAFLD group (Table 1).

### Comparison of subjects who developed NAFLD with non-NAFLD subjects

Two hundred sixty-six (17.1%) patients from the non-NAFLD group in 1998 were newly diagnosed with NAFLD in 2008 (164 men 21.2%, 102 women 12.9%) (Fig. 1). The baseline characteristics in 1998 were compared between the new-NAFLD and non-NAFLD groups. Age, frequency of male gender, obesity, dyslipidemia, and cigarette smoking differed significantly between the two groups (Table 2). These factors also had an independent association with NAFLD development (all subjects in Table 3), indicating that smokers were likely to develop NAFLD.

Furthermore, in a limited group of subjects that excluded former smokers (before 1998), new smokers after 1998 and those who quit between 1998 and 2008 (new quitters), cigarette smoking tended to be a risk factor for NAFLD development [adjusted odds ratio (AOR) 1.44, 95% CI 0.86–2.42 among the limited group of subjects in Table 3].

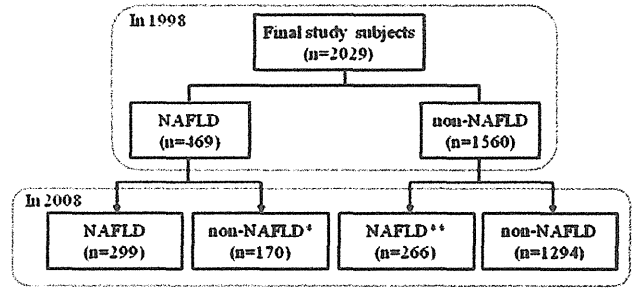


**Fig. 1** Study flow diagram. A total of 2,029 subjects were enrolled in the study. * One hundred seventy subjects had apparent nonalcoholic fatty liver disease (NAFLD) in 1998, but not in 2008. ** Two hundred sixty-six subjects were newly diagnosed with NAFLD in 2008

### Association between cigarette smoking and the development of NAFLD

The association between smoking patterns and NAFLD was analyzed by classifying the subjects into 3 groups: BI = 0 (non-smokers), BI = 1–399, and BI ≥400. Of the 1,553 subjects in the non-NAFLD group in 1998 (excluding 7 subjects whose BI was not calculated because of lack of data), the risk of developing NAFLD correlated positively with BI in multivariate analysis adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia, and alcohol intake (Table 4).

### Association of metabolic syndrome risk factors and cigarette smoking with NAFLD development

The association of obesity, hypertension, dyslipidemia, and dysglycemia (four metabolic syndrome risk factors) with the incidence of NAFLD was analyzed. As shown in Fig. 2, the incidence of NAFLD was 13.2% in subjects with no metabolic syndrome risk factors, and 19.0, 22.5, and 24.7% in those with 1, 2 and ≥3 factors, respectively. The risk of NAFLD was significantly correlated with number of metabolic syndrome risk factors (Table 5).

**Table 2** Comparison of baseline characteristics in subjects with development or regression of nonalcoholic fatty liver disease

| Characteristic | Non-NAFLD at baseline and NAFLD at follow-up ($n$ = 266) | Non-NAFLD at baseline and follow-up ($n$ = 1,294) | $P$ value[a] | NAFLD at baseline and non-NAFLD at follow-up ($n$ = 170) | NAFLD at baseline and follow-up ($n$ = 299) | $P$ value[a] |
|---|---|---|---|---|---|---|
| Mean age, year ± SD | 48.0 ± 8.0 | 51.8 ± 9.4 | <0.001 | 51.6 ± 8.5 | 47.8 ± 8.8 | <0.001 |
| Men, $n$ (%) | 164 (61.7) | 608 (47.0) | <0.001 | 116 (68.2) | 226 (75.6) | 0.10 |
| BMI ≥25 kg/m$^2$, $n$ (%) | 79 (29.7) | 172 (13.3) | <0.001 | 92 (54.1) | 184 (61.5) | 0.12 |
| Hypertension, $n$ (%)[b] | 65 (24.4) | 357 (27.6) | 0.32 | 81 (47.6) | 117 (39.1) | 0.08 |
| Dyslipidemia, $n$ (%)[b] | 61 (22.9) | 155 (12.0) | <0.001 | 64 (37.6) | 136 (45.5) | 0.12 |
| Dysglycemia, $n$ (%)[b] | 57 (21.4) | 265 (20.5) | 0.74 | 72 (42.4) | 113 (37.8) | 0.38 |
| Current smoker, $n$ (%) | 94 (35.3) | 221 (17.1) | <0.001 | 38 (22.4) | 92 (30.8) | 0.05 |
| Light alcohol drinker, $n$ (%)[b] | 162 (60.9) | 743 (57.4) | 0.31 | 110 (64.7) | 200 (66.9) | 0.68 |

*NAFLD* nonalcoholic fatty liver disease, *BMI* body mass index

[a] Calculated by Fisher's exact test for categorical variables or an unpaired $t$ test for continuous variables at baseline

[b] Definitions are provided in "Subjects and methods"

**Table 3** Risk factors for the development of nonalcoholic fatty liver disease

| Variable | All subjects ($n$ = 1,560) | | | | Limited group of subjects ($n$ = 1,174)[a] | | | |
|---|---|---|---|---|---|---|---|---|
| | Unadjusted odds ratio | 95% CI | Adjusted odds ratio[b] | 95% CI[b] | Unadjusted odds ratio | 95% CI | Adjusted odds ratio[b] | 95% CI[b] |
| Age | 0.95 | 0.94–0.97 | 0.95 | 0.94–0.97 | 0.94 | 0.92–0.96 | 0.93 | 0.91–0.95 |
| Male sex | 1.81 | 1.38–2.38 | 1.46 | 1.01–2.10 | 1.62 | 1.18–2.24 | 1.28 | 0.79–2.08 |
| Obesity[c] | 2.76 | 2.03–3.75 | 3.08 | 2.20–4.32 | 3.42 | 2.38–4.91 | 4.03 | 2.69–6.06 |
| Hypertension[c] | 0.85 | 0.63–1.15 | 0.90 | 0.64–1.27 | 0.87 | 0.60–1.27 | 0.87 | 0.56–1.36 |
| Dyslipidemia[c] | 2.19 | 1.57–3.05 | 1.79 | 1.25–2.58 | 2.76 | 1.84–4.13 | 2.67 | 1.70–4.21 |
| Dysglycemia[c] | 1.06 | 0.77–1.46 | 1.04 | 0.73–1.48 | 1.28 | 0.87–1.89 | 1.41 | 0.92–2.16 |
| Cigarette smoking | 2.65 | 1.98–3.54 | 1.91 | 1.34–2.72 | 2.29 | 1.57–3.35 | 1.44 | 0.86–2.42 |
| Light alcohol intake[c] | 1.16 | 0.88–1.51 | 0.75 | 0.54–1.04 | 1.03 | 0.75–1.41 | 0.61 | 0.41–0.92 |

*CI* Confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

[c] Definitions are provided in "Subjects and methods"

**Table 4** Incidence of nonalcoholic fatty liver disease based on smoking status in 1998

| Brinkman index[a] | Newly diagnosed NAFLD ($n$ = 265), $n$ (%)[b] | Non-NAFLD ($n$ = 1,288) $n$ (%)[b] | $P$ value[c] | Unadjusted odds ratio (95% CI) | Adjusted odds ratio (95% CI)[d] |
|---|---|---|---|---|---|
| 0 | 172 (64.9) | 1,072 (83.2) | | 1.0 | 1.0 |
| 1–399 | 24 (9.1) | 57 (4.4) | <0.001 | 2.62 (1.59–4.34) | 1.77 (1.02–3.07) |
| ≥400 | 69 (26.0) | 159 (12.3) | | 2.70 (1.95–3.74) | 2.04 (1.37–3.03) |

*NAFLD* nonalcoholic fatty liver disease, *CI* confidence interval

[a] Calculated as the number of cigarettes smoked per day multiplied by the number of years that the subject smoked

[b] Seven subjects whose Brinkman indices were not calculated because of a lack of data were excluded from this analysis

[c] Calculated by Fisher's exact test for categorical variables

[d] Adjusted for age, sex, obesity, hypertension, dyslipidemia, dysglycemia and alcohol intake

Cigarette smoking at baseline was also found to be an independent risk factor for NAFLD development in this model (all subjects in Table 5).

The incidence of NAFLD increased as the number of metabolic syndrome risk factors increased in nonsmokers (Fig. 2). In contrast, the incidence in smokers with one or more metabolic syndrome risk factors (≥35%) was higher than in those with none, but did not differ among those with 1, 2, or ≥3. The incidence of NAFLD was significantly higher in smokers than in nonsmokers, regardless of the number of metabolic syndrome risk factors.

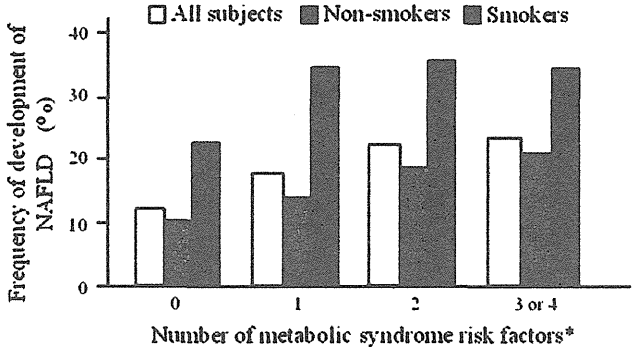


**Fig. 2** Frequency of the development of nonalcoholic fatty liver disease (NAFLD) for subjects with different numbers of metabolic syndrome risk factors in smokers and nonsmokers. The incidence of NAFLD was higher in smokers than in nonsmokers. The incidence of NAFLD in smokers with one or more metabolic syndrome risk factor was higher than in those with none, but did not differ among those with 1, 2, or ≥3 factors. * Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

Furthermore, in the limited group of subjects including continuing smokers and nonsmokers only, cigarette smoking at baseline was a risk factor for NAFLD development, although it did not achieve statistical significance (<0.05) in multivariate analysis (AOR 1.64, 95% CI 0.99–2.72 among the limited group of subjects in Table 5).

## Comparison of subjects in whom NAFLD was cured with those with persistent NAFLD

Of the 469 subjects in the NAFLD group in 1998, NAFLD was cured in 170 (36.2%) in 2008 (116 men 33.9%, 54 women 42.5%) (Fig. 1). A comparison of baseline characteristics in 1998 between the NAFLD-cured and NAFLD-persistent groups showed a significant difference in age, but not in sex, obesity, hypertension, dyslipidemia, dysglycemia, cigarette smoking, or light alcohol intake (Table 2). In multivariate analysis using these factors, only age had an independent association with cure of NAFLD. The frequency of NAFLD cure was 31.7% in subjects with no metabolic syndrome risk factors, compared with 36.1, 42.7, and 31.4% in those with 1, 2 and ≥3 factors, respectively, showing no association between NAFLD cure and the number of metabolic syndrome risk factors ($P = 0.21$).

**Table 5** Association of the development of nonalcoholic fatty liver disease and the number of metabolic syndrome risk factors

| Variables | Newly diagnosed NAFLD vs. non-NAFLD | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | All subjects ($n$ = 1,560) | | | | Limited group of subjects ($n$ = 1,174)[a] | | | |
| | Unadjusted odds ratio | 95% CI | Adjusted odds ratio[b] | 95% CI[b] | Unadjusted odds ratio | 95% CI | Adjusted odds ratio[b] | 95% CI[b] |
| Age | 0.95 | 0.94–0.97 | 0.95 | 0.93–0.96 | 0.94 | 0.92–0.96 | 0.92 | 0.90–0.94 |
| Male sex | 1.81 | 1.38–2.38 | 1.28 | 0.90–1.83 | 1.62 | 1.18–2.24 | 1.17 | 0.73–1.86 |
| MS risk factors | | | | | | | | |
| 0 | 1.0 | | 1.0 | | 1.0 | | 1.0 | |
| 1 | 1.55 | 1.14–2.11 | 1.86 | 1.34–2.58 | 1.93 | 1.32–2.82 | 2.47 | 1.65–3.70 |
| 2 | 1.92 | 1.33–2.77 | 2.63 | 1.77–3.92 | 2.70 | 1.73–4.21 | 4.65 | 2.83–7.64 |
| 3 or 4 | 2.16 | 1.22–3.83 | 2.99 | 1.62–5.50 | 3.15 | 1.55–6.41 | 5.20 | 2.41–11.22 |
| Cigarette smoking | 2.65 | 1.98–3.54 | 2.11 | 1.49–3.00 | 2.29 | 1.57–3.35 | 1.64 | 0.99–2.72 |
| Light alcohol intake | 1.16 | 0.88–1.51 | 0.71 | 0.51–0.97 | 1.03 | 0.74–1.41 | 0.55 | 0.37–0.81 |

Metabolic syndrome risk factors are obesity, hypertension, dyslipidemia and dysglycemia, as defined in "Subjects and methods"

*MS* metabolic syndrome, *NAFLD* nonalcoholic fatty liver disease, *CI* confidence interval

[a] Including only subjects who smoked consistently between 1998 and 2008 or those who did not smoke at all during this time

[b] Adjusted for all variables in the table

**Table 6** The frequency of NAFLD in three groups categorized by smoking status

| | Continuing nonsmokers ($n$ = 1,237)[a] | Cigarette smoker in 1998 | | $P$ value |
|---|---|---|---|---|
| | | New quitters ($n$ = 238)[b] | Continuing smokers ($n$ = 263) | |
| In 1998, $n$ (%) | 246 (19.9) | 61 (25.6) | 80 (30.4) | <0.001[c] |
| In 2008, $n$ (%) | 275 (22.2) | 91 (38.2) | 106 (40.3) | <0.001[c] |
| 1998–2008, $n$ (%) | | | | |
| NAFLD–NAFLD | 145 (11.7) | 37 (15.5) | 59 (22.4) | |
| NAFLD–non NAFLD | 101 (8.2) | 24 (10.1) | 21 (8.0) | <0.001[d] |
| Non NAFLD–NAFLD | 130 (10.5) | 54 (22.7) | 47 (17.9) | [0.16][e] |
| Non NAFLD–non NAFLD | 861 (69.6) | 123 (51.7) | 136 (51.7) | |

*NAFLD* nonalcoholic fatty liver disease

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Calculated by Fisher's exact test for categorical variables

[d] Calculated by Pearson's chi-square test for categorical variables

[e] Calculated among new quitters and continuing smokers by Pearson's chi-square test for categorical variables

**Table 7** Development of NAFLD in the three groups categorized

| | Newly diagnosed NAFLD vs. non-NAFLD | | |
|---|---|---|---|
| | Continuing nonsmokers[a] Odds ratio | New quitters[b] Odds ratio (95% CI) | Continuing smokers Odds ratio (95% CI) |
| Unadjusted | 1.0 | 2.91 (2.01–4.21) | 2.29 (1.57–3.35) |
| Model 1[c] | 1.0 | 2.96 (2.00–4.38) | 1.61 (1.07–2.43) |
| Model 2[d] | 1.0 | 2.77 (1.75–4.40) | 1.50 (0.92–2.44) |
| Model 3[e] | 1.0 | 2.73 (1.71–4.36) | 1.47 (0.90–2.42) |
| Model 4[f] | 1.0 | 1.94 (1.30–2.90) | 1.91 (1.28–2.84) |

*NAFLD* nonalcoholic fatty liver disease, *CI* confidence interval

[a] Subjects who never smoked consistently from 1998 to 2008

[b] Smokers in 1998 but stopped smoking in 2008

[c] Adjusted for age, obesity, and dyslipidemia

[d] Adjusted for all factors in model 1 plus sex

[e] Adjusted for all factors in model 2 plus hypertension, dysglycemia and light alcohol intake

[f] Adjusted for increase of body mass index (BMI) during the 10-year period

### Influence of smoking cessation on NAFLD status or NAFLD development

The association between smoking cessation and risk of NAFLD was analyzed using subjects who never smoked, those who smoked consistently from 1998 to 2008, and new quitters who were smokers in 1998 but had stopped by 2008. The frequency of NAFLD cure in new quitters was similar (10.1%) to those in continuing smokers (8.0%) and nonsmokers (8.2%, Table 6). In contrast, the frequency of the development of NAFLD in new quitters (22.7%) was higher than that in nonsmokers (10.5%) and was similar to that in continuing smokers (17.9%). Furthermore, after adjusting for age, obesity, dyslipidemia, sex, hypertension, dysglycemia and alcohol intake, compared with nonsmokers, the odds ratios of the development of NAFLD among new quitters and continuing smokers were 2.73 (95% CI 1.71–4.36) and 1.47 (95% CI 0.90–2.42), respectively (Table 7). In addition, after adjusting for an increase in BMI from 1998 to 2008 (Table 7, model 4), the odds ratio in new quitters decreased more compared to that in continuing smokers (2.91–1.94 vs. 2.29–1.91).

## Discussion

During the 10-year period of the study, 17.1% of the subjects developed NAFLD. Cigarette smoking was an

independent risk factor for NAFLD, in addition to age, obesity, dyslipidemia, and the total number of metabolic syndrome risk factors. The Brinkman index (a smoking index) was also associated with NAFLD development. Metabolic syndrome risk factors are known to be related to NAFLD, but this is the first follow-up study over a 10-year period to show that cigarette smoking is an independent risk factor for NAFLD development, as well as for metabolic syndrome risk factors. However, in multivariate analysis, the association between cigarette smoking and NAFLD development did not reach statistical significance in the limited group of subjects, which may be due in part to the modest sample size. In addition, subanalysis using subjects who quit smoking demonstrated that smoking cessation seems to be a risk for NAFLD development, a result which can be partially explained by an increase in BMI.

Cigarette smoking had been previously associated with chronic liver diseases such as chronic hepatitis C and B, primary biliary cirrhosis, and alcoholic liver diseases [23–25], but the association between NAFLD and cigarette smoking had not been fully elucidated. Suzuki et al. [26] reported that initiation of cigarette smoking in patients with NAFLD was associated with ALT elevation in a 1-year follow-up survey, but the association between the development of NAFLD and cigarette smoking was not fully investigated. Chavez-Tapia et al. [27] showed that smoking was not associated with NAFLD in univariate regression analysis, but found that the risk of NAFLD tended to increase in subjects who smoked $\geq$10 (OR = 1.16 [95% CI 0.76–1.64]) and $\geq$20 (OR = 1.54 [95% CI 0.94–2.52]) packs per year compared to nonsmokers. These results may depend on the number of subjects and the duration of the study, and require confirmation in larger long-term longitudinal studies.

Increases in BMI partially explained the excess risk of NAFLD development in smokers in 1998, especially in new quitters (Table 7). Other mechanisms are also speculated, for instance smoking-induced fatty changes and fibrosis in the liver [28–40]. $H_2O_2$ and nicotine produced by smoking reduce adiponectin expression [30, 31]. Smoking also promotes the production of activated NADPH oxidase-induced reactive oxygen species, which enhances oxidative stress and lipid peroxidation due to impaired antioxidative action [32–34]. Yuan et al. [35] reported that cigarette smoking inactivated 5′-adenosine monophosphate-activated protein kinase (AMPK) by dephosphorylation and promoted triglyceride accumulation in hepatocytes via activation of sterol regulatory element-binding protein-1 (SREBP-1), inducing fatty liver in mice fed a high-fat diet. In heavy smokers, tissue becomes hypoxic due to elevation of carbon monoxide and hemoglobin levels and impairment of oxygen transport by red blood cells, which induces erythropoietin production and promotes iron absorption in the intestines [36]. Excess iron is thought to be deposited in the liver and to eventually induce hepatocellular damage and fibrosis [36]. In addition, oxidative stress produces necrotizing inflammation in fatty liver [37]. In obese rats, cigarette smoking elevated ALT and caused hepatocellular ballooning and lobular inflammation [38]. Smoking also promotes the production of inflammatory cytokines and hepatic fibrosis-associated molecules [38–40]. Further investigation of the mechanism whereby smoking influences development or progression of NAFLD in humans is required.

There is a sex difference in the incidence of NAFLD, with men being more likely to develop fatty liver compared to women [7, 41, 42]. A similar result was obtained in this study. The prevalence of fatty liver has been shown to be about 25% in men in their 30s–60s, while it gradually increases with age in women and reaches a similar prevalence after 60 years of age [7]. Sex hormones are involved in this change, and postmenopausal reduction of estrogen levels is thought to promote visceral fat accumulation and induce insulin resistance [43]. The involvement of smoking in increasing testosterone levels has been proposed [44], suggesting that sex hormones are involved in cigarette smoking-induced NAFLD [45]. Therefore, smoking and changes in sex hormones may both be related to the development or progression of NAFLD.

Many cross-sectional studies have shown that metabolic syndrome risk factors are associated with NAFLD [3, 4, 7–9], but causal relationships cannot be proven by cross-sectional studies alone. Associations of changes in body weight and metabolic syndrome with the development and cure of NAFLD have been demonstrated in longitudinal studies [19, 26, 46, 47]. Hamaguchi et al. [19] followed 4,401 healthy subjects for an average period of 414 days and found that new NAFLD developed in 308 subjects (10% of the non-NAFLD subjects). The presence of metabolic syndrome was most strongly associated with newly developed NAFLD, and body weight gain was also an independent risk factor. NAFLD was cured in 113 subjects (16% of the NAFLD patients) during the observation period, with body weight loss being the most important factor, indicating that weight loss is more important than the absence of metabolic syndrome. In our study, the incidence of NAFLD development after 10 years was investigated based on the number of metabolic syndrome risk factors in 1998, and was found to increase as the number of risk factors increased (Fig. 2; Table 5). In addition, the number of metabolic syndrome risk factors in 1998 was not associated with cure of NAFLD after 10 years. Body weight loss during the 10-year period was an independent factor contributing to NAFLD cure (data not shown), similar to the findings of Hamaguchi et al. [19]. In contrast, in