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【 ORIGINAL ARTICLE 】

Predictors of Insulin Secretion in Japanese Patients with Histopathologically-confirmed Non-alcoholic Fatty Liver Disease

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Abstract:

Objective The correlation between the insulin secretion levels and the risk of hepatocarcinogenesis is clinically important. The aim of the present study was to determine the effects of various clinical parameters on C-peptide (CPR) levels in patients with non-alcoholic fatty liver disease (NAFLD).

Methods In this retrospective cohort study, the effects of clinical parameters on insulin resistance (HOMA-IR) and insulin secretion levels (HOMA-β and fasting CPR) were investigated.

Patients A total of 244 Japanese patients with histopathologically confirmed NAFLD were evaluated. Of these, 77 underwent the meal tolerance test (MTT) to evaluate the association of various clinical parameters with the CPR levels at 120 minutes.

Results A multivariate analysis identified fasting plasma glucose (FPG) (≥110 mg/dL), aspartate aminotransferase (≥1.0×ULN IU/L), and a large waist circumference as independent predictors of insulin resistance (HOMA-IR ≥2.5) or high fasting CPR levels. Significant parameters for a low insulin secretion capacity (HOMA-β <30%) were not detected, except for the parameters mentioned in the diagnostic criteria of diabetes mellitus. Regarding the MTT, the CPR levels at 120 minutes were significantly higher in patients with fibrosis stage 3-4 than in those with stage 0-2. Body composition and genetic variation did not affect the CPR levels at 120 minutes. A multivariate analysis identified fibrosis stage (3-4), hyperuricemia, FPG (≥ 110 mg/dL), and procollagen III peptide (>1.0 U/mL) as independent predictors of high CPR levels at 120 minutes.

Conclusion The present study showed that high plasma glucose levels and severe liver fibrosis stage influence insulin secretion levels in Japanese patients with NAFLD. Conservation of delayed insulin secretion levels was confirmed in patients with severe liver fibrosis.

Key words: nonalcoholic fatty liver disease, nonalcoholic steatohepatitis, fibrosis stage, C-peptide, HOMA-IR, HOMA-β

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Introduction

Non-alcoholic fatty liver disease (NAFLD) is a common liver disease worldwide (1-4) and associated with serious complications. NAFLD encompasses a wide spectrum of liver pathologies, including benign non-alcoholic fatty liver and non-alcoholic steatohepatitis (NASH). In certain patients free of a history of excessive alcohol intake, NASH can progress gradually to liver cirrhosis, hepatocellular carcinoma

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(HCC), and liver failure (5). In this regard, various treatments are reported to improve the histopathological features of NAFLD (6-8).

Evidence suggests the presence of a peculiar relationship between glucose metabolism and the risk of hepatocarcinogenesis. Previous studies have reported that hyperinsulinemia correlates negatively with the clinical course of HCC (9-12). Kawaguchi et al. (9) highlighted the correlation of exogenous insulin or treatment with sulphonylurea and the likelihood of HCC in patients with hepatitis C virus (HCV) infection. Others discussed the possible effects of branched-chain amino acids on insulin resistance and HCC in patients with chronic liver diseases (12). Another study described the correlation between the baseline serum levels of C-peptide (CPR), which is released from proinsulin during β-cell insulin secretion, and an increased risk of HCC, independent of obesity and other HCC risk factors (13). Thus, there is a clinical need to analyze the insulin secretion levels for the early identification of HCC-high-risk groups. However, no detailed information is available on the relationship between the clinical features of NAFLD (e.g. histological features, body composition, and genetic variation) and glucose metabolism, including insulin resistance and the insulin secretion capacity.

The purpose of this study was to determine the impact of clinical parameters on the CPR levels as the indicator of insulin secretion levels and the early predictor of HCC-high-risk groups in Japanese patients with histopathologically-confirmed NAFLD.

Materials and Methods

Patients

In this retrospective study, the effects of various clinical parameters on the insulin resistance represented by homeostatis model assessment of insulin resistance (HOMA-IR), and the insulin secretion capacity, represented by HOMA-β, were investigated in 244 of 436 Japanese patients diagnosed with NAFLD histologically from January 1980 to February 2019 at Toranomon Hospital (Table 1). None had had antidiabetic agents with insulin preparations introduced. The effects of various clinical parameters on the insulin secretion levels, represented by fasting CPR levels, were also investigated in 100 of the 244 Japanese patients. Furthermore, the association among various clinical parameters and CPR levels measured at 120 minutes [based on the meal tolerance test (MTT)] was examined in 77 of the 100 patients.

NAFLD was diagnosed based on the histopathological findings of a liver biopsy, consisting of steatosis in ≥5% of hepatocytes and absence of autoimmune hepatitis, viral hepatitis, drug-induced liver disease, primary biliary cholangitis, hemochromatosis, biliary obstruction, α-1-antitrypsin deficiency-associated liver disease, and Wilson disease. Patients who consumed more than 20 g/day alcohol were excluded.

The study protocol was approved by the Human Ethics Review Committee at Toranomon Hospital, and each patient gave their signed informed consent at the time of the liver histological diagnosis. The study was conducted in compliance with the International Conference on Harmonisation guidelines for Good Clinical Practice (E6) and the 2013 Declaration of Helsinki.

Liver histopathology

Adequate liver biopsy specimens (at least 1.5 cm long containing a minimum of 11 portal tracts) were obtained using a 14-gauge modified Vim Silverman needle (Tohoku University style; Kakinuma Factory, Tokyo, Japan) and then fixed in 10% formalin, cut into thin sections, and stained with hematoxylin-eosin, Masson trichrome, silver impregnation, and periodic acid-Schiff after digestion with diastase.

A panel of four pathologists (F.K., T.F., T.F., and K.K.) blinded to the clinical information established the final histopathological diagnosis by consensus. Specimens were graded for hepatocyte steatosis into grades 0, 1, 2, and 3, which represented <5%, 5-33%, >33-66%, and >66% steatosis, respectively. Lobular inflammation was scored as 0, 1, 2, and 3, representing no foci, <2 foci, 2-4 foci, and >4 foci per 200× field, respectively. Hepatocyte ballooning was scored as 0, 1, and 2, reflecting none, few cells, and many cells, respectively. NAFLD activity score (NAS, range, 0-8 points) represented the sum of the scores of steatosis, lobular inflammation, and hepatocyte ballooning, as described previously by Kleiner et al. (14). Fibrosis was scored as stage 0, 1, 2, 3, and 4, which represented none, zone 3 perisinusoidal fibrosis, zone 3 perisinusoidal fibrosis with portal fibrosis, zone 3 perisinusoidal fibrosis, portal fibrosis, and bridging fibrosis, and cirrhosis, respectively (14, 15). NASH was defined according to the Fatty Liver Inhibition of Progression (FLIP) algorithm (16).

MTT

Each subject underwent the MTT, which was used to evaluate the amount of insulin secreted. The test involved the consumption of 500 kcal of a typical Japanese breakfast, followed by the measurement of the plasma glucose and CPR levels at 60 and 120 minutes, as described in detail previously (17-19).

Clinical parameters

The following normal range values of our hospital were used for the assessments: aspartate aminotransferase (AST), 13-33 IU/L (men) and 6-27 IU/L (women); alanine aminotransferase (ALT), 8-42 IU/L. Type 2 diabetes mellitus (T2DM) was diagnosed in cases with a high fasting plasma glucose (FPG, ≥126 mg/dL), high hemoglobin A1c (HbA1c, ≥6.5%), use of glucose-lowering agents, or a self-reported history of a clinical diagnosis. Obesity was defined as a body mass index (BMI) of more than 25.0 kg/m^2. A normal waist circumference (WC) was defined as 85 cm for men and 90 cm for women.

Table 1. Patient Characteristics at the Time of Histological Diagnosis of NAFLD.

Demographic data	
Numbers of patients	244
Gender, Male/Female, n	144/100
Age, years*	55 (20-85)
Type 2 diabetes mellitus, No/Yes, n	141/103
Hypertension, No/Yes, n	131/113
Hyperlipidemia, No/Yes, n	133/111
Hyperuricemia, No/Yes, n	212/32
Smoking, No/Yes, n	207/37
Histological findings	
Steatosis, 5-33%/>33-66%/>66%, n	84/95/65
Lobular inflammation	
No foci/<2 foci/2-4 foci/>4 foci per 200×field, n	6/137/91/10
Ballooning, None/Few cells/Many cells, n	12/179/53
Stage, 0/1/2/3/4, n	14/102/39/74/15
NAFLD activity score, ≤2/3, 4/≥5, n	10/109/125
Diagnosis according to FLIP algorithm, NASH /non-NASH, n	229/10
Genetic variation	
PNPLA3 rs738409 (CC/CG/GG/Not determined)	38/79/76/51
TM6SF2 rs58542926 (CC/CT/TT/Not determined)	152/37/4/51
Laboratory data*	
Serum aspartate aminotransferase, IU/L	41 (10-378)
Serum alanine aminotransferase, IU/L	63 (9-783)
Gamma-glutamyl transpeptidase, IU/L	59 (11-659)
Albumin, g/dL	4.2 (3.0-5.4)
Estimate glomerular filtration rate, mL/min/1.73m^3	81.9 (27.9-147.9)
Leukocyte count, /mm^3	5,800 (3,000-13,400)
Hemoglobin, g/dL	14.9 (9.2-18.7)
Platelet count,×10^3/mm^3	210 (50-377)
Fasting plasma glucose, mg/dL	108 (78-287)
Hemoglobin A1c, %	6.2 (4.3-12.2)
Fasting immunoreactive insulin, μU/mL	13 (2-83)
Fasting C-peptide, ng/mL	2.49 (1.18-5.62)
HOMA-IR	3.4 (0.5-36.4)
HOMAβ, %	98 (10-228)
Uric acid, mg/dL	5.8 (2.6-10.7)
Total cholesterol, mg/dL	191 (101-317)
Triglycerides, mg/dL	135 (31-610)
High-density lipoprotein cholesterol, mg/dL	45 (21-86)
Low-density lipoprotein cholesterol, mg/dL	108 (28-227)
Serum ferritin, μg/L	207 (<10-2,067)
Hyaluronic acid, μg/L	34 (5-814)
High sensitive C-reactive protein, mg/dL	0.086 (0.004-1.356)
Type IV collagen 7S, ng/mL	4.6 (1.9-21.2)
Procollagen III peptide, U/mL	0.70 (0.40-1.90)
Alpha-fetoprotein, μg/L	4 (1-20)
Body composition based on bioelectrical impedance analysis	
Body mass index, kg/m^2	26.7 (19.1-41.6)
Waist circumference, cm	91.8 (69.9-126.6)
Skeletal muscle mass index (SMI)	7.47 (4.26-10.43)
Sarcopenia diagnosis according to SMI, No/Yes/unknown, n	131/14/99
Sarcopenia index (SI)	0.76 (0.34-1.27)
Sarcopenia diagnosis according to SI, No/Yes/unknown , n	103/43/98

Data are number of patients, except those denoted by *, which represent the median (range) values.

Insulin resistance and insulin secretion capacity

Insulin resistance was defined as HOMA-IR≥2.5, and a low insulin secretion capacity was defined as HOMA-β< 30% (20).

Determination of the PNPLA3 and TM6SF2 genotypes

The TaqMan SNP genotyping assay (Applied Biosystems, Foster City, USA) was used for genotyping *PNPLA3* rs 738409 and *TM6SF2* rs58542926.

Body composition and the definition of sarcopenia

A bioelectrical impedance analysis performed with an Inbody770 multifrequency impedance body composition analyzer (Inbody Japan, Tokyo, Japan) was used to assess the body composition. The skeletal muscle mass index (SMI) represented the skeletal muscle mass of both arms and legs, expressed as (kg)/[height (m)]^2. Based on the diagnostic criteria of sarcopenia by the Japan Society of Hepatology (21), a low SMI was defined as <7.0 kg/m^2 for men and <5.7 kg/m^2 for women. We also calculated the sarcopenia index (SI) as the appendicular skeletal muscle mass (kg) divided by the BMI (kg/m^2) and divided patients into those with and without sarcopenia, using a cut-off SI of 0.789 for men and < 0.521 for women, according to the NIH Sarcopenia Project (22).

Statistical analyses

The relationship between the clinical features determined at the diagnosis (Table 1) and the surrogate markers of glucose metabolism (e.g. HOMA-IR, HOMA-β, and CPR levels) were analyzed using non-parametric tests, including the chi-squared test, Mann-Whitney U test, Fisher's exact probability test, and Kruskal-Wallis test, as required. Uni- and multivariate logistic regression analyses were also applied to identify the clinical parameters that correlated significantly with the surrogate markers of glucose metabolism. The odds ratios (ORs) and 95% confidence intervals (CIs) were also calculated. p<0.05 by the two-tailed test denoted the presence of significant differences. Variables that achieved statistical significance (p<0.05) on a univariate analysis were entered into the multiple logistic regression analysis after being converted into categorical data consisting of two simple ordinal numbers. The SPSS software program (SPSS Inc., Chicago, USA) was used for all statistical analyses.

Results

Predictors of high HOMA-IR values

Insulin resistance was evaluated as HOMA-IR ≥2.5. Table 2 shows the predictive factors associated with insulin resistance. A univariate analysis showed close relationships between insulin resistance and the age (≥70 years; p=0.016), lobular inflammation (≥2 foci; p=0.001), stage (≥2; p=0.003), type 2 diabetes mellitus (p<0.001), hypertension (p=0.001), hyperuricemia (p=0.035), FPG (≥110 mg/dL; p<0.001), HbA1c (≥5.8%; p<0.001), AST [≥1.0× upper limit of normal (ULN) IU/L; p<0.001], ALT (≥1.5×ULN IU/L; p<0.001), platelet count (≥200×10^3/mm^3; p=0.001), highly-sensitive C-reactive protein (>0.2 mg/dL; p=0.043), hyaluronic acid (≥51 μg/L; p=0.015), PIIIP (>1.0 U/mL; p=0.001), type IV collagen 7S (>6.0 ng/mL; p=0.001), BMI (≥27.0 kg/m^2; p<0.001), WC (large; p<0.001), and sarcopenia (p=0.006). The above parameters were entered into a multivariate analysis, which identified a high FPG (≥110 mg/dL; OR 5.99 p=0.002), high AST (≥1.0×ULN IU/L; OR 4.97, p=0.004), and large WC (OR 3.72, p=0.016) as significant and independent predictors of insulin resistance.

Predictors of low HOMA-β values

A low insulin secretion capacity was evaluated as HOMA-β <30%. A univariate analysis showed close relationships between insulin secretion capacity and type 2 diabetes mellitus (p=0.010), FPG (≥126 mg/dL; p=0.001), and HbA1c (≥6.5%; p=0.003). No significant parameters were detected, except for the parameters included in the diagnostic criteria of type 2 diabetes mellitus.

Predictors of high levels of fasting CPR

The median level of fasting CPR for the entire group was 2.6 ng/mL. Table 3 shows the predictive factors associated with high levels (≥2.6 ng/mL) of fasting CPR. A univariate analysis showed close relationships between CPR and steatosis (>66%; p=0.025), hyperuricemia (p=0.039), FPG (≥110 mg/dL; p=0.005), HbA1c (≥5.8%; p=0.014), AST (≥1.0× ULN IU/L; p=0.002), ALT (≥1.5×ULN IU/L; p<0.001), highly-sensitive C-reactive protein (>0.2 mg/dL; p=0.023), PIIIP (>1.0 U/mL; p=0.010), type IV collagen 7S (>6.0 ng/mL; p=0.013), BMI (≥27.0 kg/m^2; p<0.001), WC (large; p<0.001), and sarcopenia (p=0.049). The above parameters were entered into a multivariate analysis, which identified a high FPG (≥110 mg/dL; OR 3.18, p=0.031), high AST (≥1.0 ×ULN IU/L; OR 5.15, p=0.003), and large WC (OR 4.42, p=0.010) as significant and independent predictors of high levels of fasting CPR. Interestingly, the predictors of high levels of fasting CPR (high FPG, high AST, and large WC) were the same as those of high HOMA-IR values.

Correlation between the CPR levels and histopathological findings, based on the MTT

The MTT was conducted in 77 patients. Fig. 1 shows the CPR levels after the MTT stratified according to the histological components of NASH. The CPR levels did not correlate with the severity of steatosis (baseline, p=0.089; 60 minutes, p=0.372; and 120 minutes, p=0.153) (Fig. 1A), extent of lobular inflammation (baseline, p=0.686; 60 minutes, p=0.971; and 120 minutes, p=0.866) (Fig. 1B), or ballooning (baseline, p=0.593; 60 minutes, p=0.676; and 120 minutes, p=0.841) (Fig. 1C). However, they did correlate with the stage of liver fibrosis, being significantly higher at 120

Table 2. Factors Associated with Insulin Resistance (HOMA-IR≥2.5).

Factors	Category	Univariate analysis	Multivariate analysis		
		p	Odds ratios	(95% confidence interval)	p
Age, years	<70				
	≥70	0.016			
Lobular inflammation	<2 foci				
	≥2 foci	0.001			
Stage	0, 1				
	≥2	0.003			
Type 2 diabetes mellitus	No				
	Yes	<0.001			
Hypertension	No				
	Yes	0.001			
Hyperuricemia	No				
	Yes	0.035			
Fasting plasma glucose, mg/dL	<110		1		
	≥110	<0.001	5.99	(1.97-18.2)	0.002
Hemoglobin A1c, %	<5.8				
	≥5.8	<0.001			
Serum aspartate aminotransferase, IU/L	<1.0×ULN*		1		
	≥1.0×ULN	<0.001	4.97	(1.67-14.8)	0.004
Serum alanine aminotransferase, IU/L	<1.5×ULN				
	≥1.5×ULN	<0.001			
Platelet count, $\times 10^3/mm^3$	<200				
	≥200	0.001			
High sensitive C-reactive protein, mg/dL	≤0.2				
	>0.2	0.043			
Hyaluronic acid, μg/L	<51				
	≥51	0.015			
Procollagen III peptide, U/mL	≤1.0				
	>1.0	0.001			
Type IV collagen 7S, ng/mL	≤6.0				
	>6.0	0.001			
Body mass index, kg/m^2	<27.0				
	≥27.0	<0.001			
Waist circumference, cm	small		1		
	large**	<0.001	3.72	(1.28-10.8)	0.016
Sarcopenia diagnosis by sarcopenia index	No				
	Yes	0.006			

*ULN: upper limit of normal

**large waist circumference was defined as ≥85 cm in men and ≥90 cm in women.

minutes in patients with stage 3-4 than in those with stage 0-2 (baseline, p=0.356; 60 minutes, p=0.068; and 120 minutes, p=0.005) (Fig. 1D).

Fig. 2 shows the CPR levels at 120 minutes according to the histopathological components of NASH (steatosis, p=0.153; lobular inflammation, p=0.866; ballooning, p=0.841; and stage, p=0.005). Taken together, the above results suggest that the extent of liver fibrosis correlates directly with the CPR level at 120 minutes.

Correlation between the CPR levels and sarcopenia, based on the MTT

There was no significant difference in the CPR levels between patients with and without sarcopenia, as defined by the SMI (baseline, p=0.350; 60 minutes, p=0.639; and 120 minutes, p=0.328) and SI (excluding the baseline levels) (baseline, p=0.009; 60 minutes, p=0.379; and 120 minutes, p=0.588). These results suggest that sarcopenia has no influence on the CPR levels at 120 minutes.

Correlation between the CPR levels and genetic variation, based on the MTT

The analysis of genetic variation of *PNPLA3* rs738409 showed no significant effect for GG alleles; there were no marked differences in CPR levels between patients with CC and CG alleles, except for 60 minutes (baseline, p=0.379; 60

Table 3. Factors Associated with High Levels of Fasting C-peptide (≥2.6 ng/mL).

Factors	Category	Univariate analysis	Multivariate analysis		
		p	Odds ratios	(95% confidence interval)	p
Steatosis	5-66%				
	>66%	0.025			
Hyperuricemia	No				
	Yes	0.039			
Fasting plasma glucose, mg/dL	<110		1		
	≥110	0.005	3.18	(1.11-9.08)	0.031
Hemoglobin A1c, %	<5.8				
	≥5.8	0.014			
Serum aspartate aminotransferase, IU/L	<1.0×ULN*		1		
	≥1.0×ULN	0.002	5.15	(1.74-15.2)	0.003
Serum alanine aminotransferase, IU/L	<1.5×ULN				
	≥1.5×ULN	<0.001			
High sensitive C-reactive protein, mg/dL	≤0.2				
	>0.2	0.023			
Procollagen III peptide, U/mL	≤1.0				
	>1.0	0.010			
Type IV collagen 7S, ng/mL	≤6.0				
	>6.0	0.013			
Body mass index, kg/m^2	<27.0				
	≥27.0	<0.001			
Waist circumference, cm	small		1		
	large**	<0.001	4.42	(1.44-13.6)	0.010
Sarcopenia diagnosis by sarcopenia index	No				
	Yes	0.049			

*ULN: upper limit of normal

**large waist circumference was defined as ≥85 cm in men and ≥90 cm in women.

minutes, p=0.024; and 120 minutes, p=0.250). Similar findings were observed with regard to *TM6SF2* rs58542926, with no significant differences in the CPR levels according to CC alleles (CT and TT alleles) (baseline, p=0.569; 60 minutes, p=0.936; and 120 minutes, p=0.968). These results show that genetic variation does not affect the CPR levels at 120 minutes.

Predictors of high levels of CPR at 120 minutes, based on the MTT

We used the median CPR level at 120 minutes (8.7 ng/mL) in this analysis. Table 4 shows the predictive factors associated with high levels of CPR at 120 minutes (≥8.7 ng/mL). A univariate analysis showed a close relationship between high CPR levels at 120 minutes and severe liver fibrosis (stage 3-4; p=0.012, PIIIP >1.0 U/mL; p=0.005), hyperuricemia (p=0.036), hyperglycemia (FPG ≥110 mg/dL; p=0.022, HbA1c ≥5.8%; p=0.032), a poor liver function (AST ≥1.5×ULN IU/L; p=0.005, ALT ≥1.0×ULN IU/L; p=0.040), and obesity (BMI ≥27.0 kg/m^2; p<0.001, large WC, p<0.001). A multivariate analysis using the above parameters identified severe liver fibrosis [3-4; OR 5.17, p=0.011, PIIIP (>1.0 U/mL); OR 25.2, p=0.002], hyperuricemia (OR 7.61, p=0.043), and hyperglycemia (FPG ≥110 mg/dL; OR 7.44, p=0.006) as significant and independent determinants of high CPR levels at 120 minutes. These results show that the stage of liver fibrosis, assessed in this study by fibrosis stage and PIIIP, affects the insulin secretion at 120 minutes.

Discussion

The present study showed that hyperglycemia (FPG ≥110 mg/dL), a poor liver function (AST ≥1.0×ULN IU/L), and obesity (large WC) significantly and independently influence insulin resistance or high fasting CPR levels. Furthermore, severe liver fibrosis (stage 3-4 and PIIIP >1.0 U/mL), hyperuricemia, and hyperglycemia were significant and independent predictors of high CPR levels at 120 minutes based on MTT. Our results are similar to those reported previously in Japanese patients with NAFLD by Kawamura et al. (3), who identified thrombocytopenia (used as a surrogate marker of fibrosis stage), diabetes, a poor liver function (high AST), and old age as risk factors of HCC (3). Taken together, the results of these studies suggest that the factors that determine high levels of fasting CPR (hyperglycemia and poor liver function) and CPR at 120 minutes (hyperglycemia and severe fibrosis) are also potentially risk factors

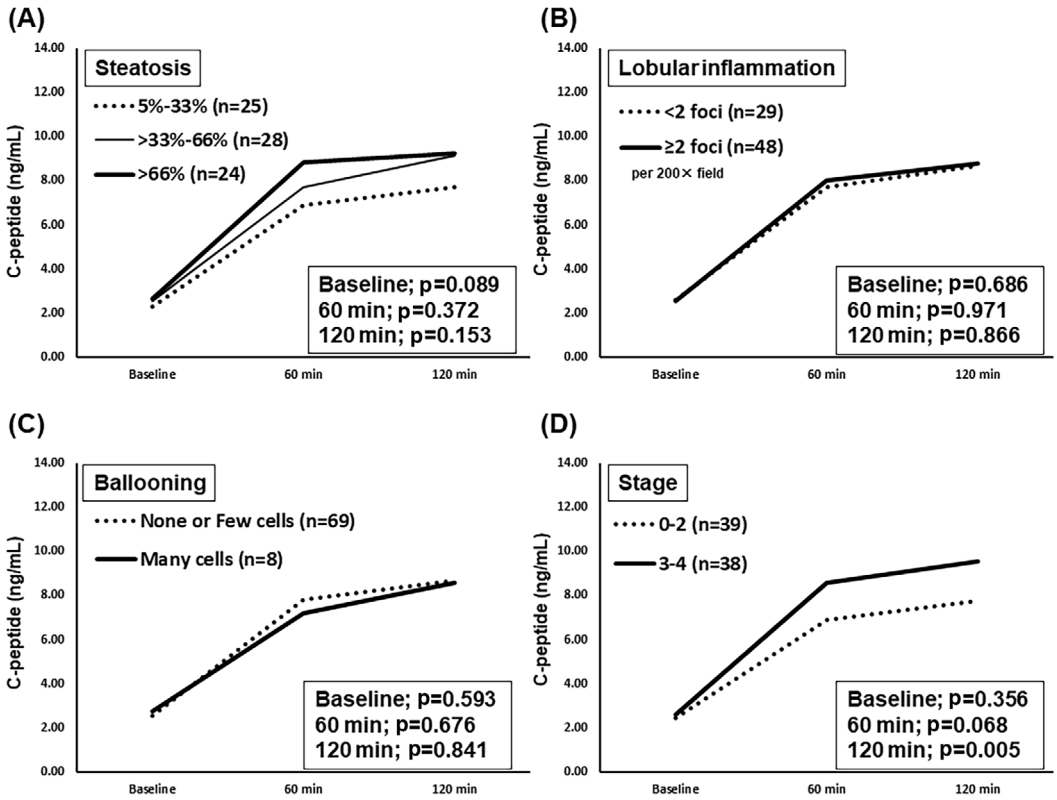

Figure 1. **C-peptide (CPR) levels at baseline, 60 min, and 120 min, based on the meal tolerance test, according to the histopathological components of NASH: (A) steatosis, (B) lobular inflammation, (C) ballooning, and (D) stage. The levels of CPR at 120 min in patients with fibrosis stage 3-4 were significantly higher than those in patients with fibrosis stage 0-2 (baseline, p=0.356; 60 min, p=0.068; and 120 min, p=0.005).**

for HCC. It is noteworthy that the body composition and genetic variation did not affect the insulin secretion levels. However, this is probably related to the small number of NAFLD patients. Further studies involving larger numbers of NAFLD patients should be performed in order to evaluate the impact of clinical parameters on the CPR levels, as the indicator of insulin secretion levels and an early predictor of HCC-high-risk groups.

In the present study, high FPG levels correlated with high CPR levels at both baseline and 120 minutes, whereas severe fibrosis stage correlated with high CPR levels at 120 minutes. Iwahashi et al. (23) previously reported that early-phase insulin secretion was higher in obese Japanese patients than in non-obese patients at all stages of glucose tolerance and that delayed insulin secretion levels were also conserved in obese Japanese patients. The present findings in patients with NAFLD indicated that early-phase insulin secretion in fibrosis stage 3-4 tended to be higher than in stage 0-2 (CPR at 60 minutes; p=0.068), and delayed insulin secretion levels were conserved in fibrosis stage 3-4 (CPR at 120 minutes; p=0.005). To our knowledge, this is the first report to show the conservation of delayed insulin secretion levels in NAFLD with severe liver fibrosis. Further studies should be conducted to investigate whether or not CPR levels before and/or after a meal affect the progression of NAFLD. The present study identified the fibrosis stage and PIIIP of fibrosis marker as independent factors. As one of the reasons, the results may indicate that PIIIP reflects not only the fibrosis stage but also inflammation, at least in part.

Previous studies have concluded that various glucose-lowering agents [e.g., pioglitazone, glucagon-like peptide-1 receptor agonists, and sodium-glucose co-transporter 2 inhibitor (SGLT2i)] can improve the histopathological features, including the fibrosis stage, in NAFLD complicated with T2DM (6, 24, 25). Another recent prospective study that used serial liver biopsies concluded that treatment with SGLT2i reduced the liver fibrosis stage in three of nine NAFLD patients; six patients had lower levels of CPR at the second biopsy than at the first biopsy, whereas the other three had higher levels at the second biopsy than at the first (26). It therefore seems that glucose-lowering agents might affect the histopathological features via their effects on glucose metabolism. However, whether or not the improvement of fibrosis affects the insulin secretion levels and HCC remains unclear. Further prospective studies are needed to investigate the effects of improving histopathological changes and glucose metabolism with anti-diabetic agents on HCC in patients with NAFLD complicated with diabetes.

The present study has certain limitations. First, the impact of the CPR level on the cumulative HCC rate could not be investigated in the present study. Second, the relationships among hyperglycemia, hyperinsulinemia, and hepatocarcino-

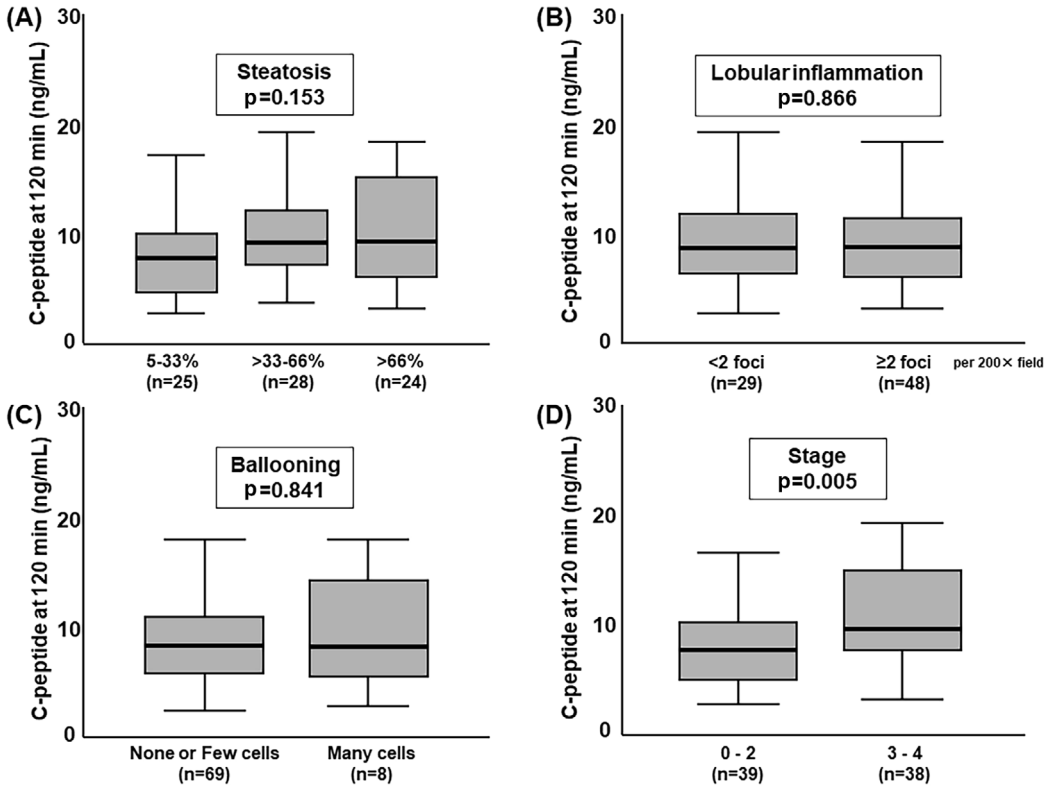

Figure 2. **C-peptide (CPR) levels at 120 min, based on the meal tolerance test, according to the individual histopathological components of NASH: (A) steatosis, (B) lobular inflammation, (C) ballooning, and (D) stage. The fibrosis stage influenced CPR levels at 120 min (p=0.005). Bars within the boxes represent the median values. The boxes denote the 25th to 75th percentiles, and the bottom and top bars represent the 10th and 90th percentiles, respectively.**

Table 4. **Factors Associated with High C-peptide Levels at 120 min (≥8.7 ng/mL), Based on Meal Tolerance Test.**

Factors	Category	Univariate analysis	Multivariate analysis		
		p	Odds ratios	(95% confidence interval)	p
Stage	0-2		1		
	3-4	0.012	5.17	(1.46-18.3)	0.011
Hyperuricemia	No		1		
	Yes	0.036	7.61	(1.07-54.2)	0.043
Fasting plasma glucose, mg/dL	<110		1		
	≥110	0.022	7.44	(1.80-30.7)	0.006
Hemoglobin A1c, %	<5.8				
	≥5.8	0.032			
Serum aspartate aminotransferase, IU/L	<1.5×ULN*				
	≥1.5×ULN	0.005			
Serum alanine aminotransferase, IU/L	<1.0×ULN				
	≥1.0×ULN	0.040			
Procollagen III peptide, U/mL	≤1.0		1		
	>1.0	0.005	25.2	(3.38-188)	0.002
Body mass index, kg/m^2	<27.0				
	≥27.0	<0.001			
Waist circumference, cm	small				
	large**	<0.001			

*ULN: upper limit of normal

**large waist circumference was defined as ≥85 cm in men and ≥90 cm in women.

genesis could not be evaluated. Furthermore, the associations of hyperinsulinemia before and/or after a meal with HCC could not be analyzed. Third, we were also unable to evaluate the impact of anti-diabetic agents without insulin preparations on hepatocarcinogenesis, as previously reported (9). Further studies are needed in order to enhance our understanding of the complex interaction among glucose metabolism, histopathological findings, and hepatocarcinogenesis, which will hopefully facilitate the design of new, effective therapies.

In conclusion, the present study investigated the impact of various clinical parameters on the CPR levels in patients with NAFLD and showed that high plasma glucose levels and a severe liver fibrosis stage affect the insulin secretion levels. Further studies are needed to determine the relationships among glucose metabolism, histopathological features, and hepatocarcinogenesis in Japanese patients with NAFLD.

The study protocol was approved by the Human Ethics Review Committee at Toranomon Hospital, and each patient gave their signed informed consent at the time of the liver histological diagnosis. The study was conducted in compliance with the International Conference on Harmonisation guidelines for Good Clinical Practice (E6) and the 2013 Declaration of Helsinki.

Author's disclosure of potential Conflicts of Interest (COI).
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