Plasma Heparin Cofactor II Activity Is Inversely Associated with Hepatic Fibrosis of Non-Alcoholic Fatty Liver Disease in Patients with Type 2 Diabetes Mellitus

Tomoyo Hara[1], Ryoko Uemoto[2], Akiko Sekine[2], Yukari Mitsui[1], Shiho Masuda[1], Hiroki Yamagami[1], Kiyoe Kurahashi[1], Sumiko Yoshida[1], Toshiki Otoda[2], Tomoyuki Yuasa[2], Akio Kuroda[3], Yasumasa Ikeda[4], Itsuro Endo[5], Soichi Honda[6], Katsuhiko Yoshimoto[7, 8], Akira Kondo[8], Toshiaki Tamaki[9], Toshio Matsumoto[10], Munehide Matsuhisa[3], Masahiro Abe[1] and Ken-ichi Aihara[2, 9]

[1] Department of Hematology, Endocrinology and Metabolism, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan
[2] Department of Community Medicine and Medical Science, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan
[3] Diabetes Therapeutics and Research Center, Institute of Advanced Medical Sciences, Tokushima University, Tokushima, Japan
[4] Department of Pharmacology, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan
[5] Department of Bioregulatory Sciences, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan
[6] Minami Municipal National Insurance Hospital, Tokushima, Japan
[7] Department of Medical Pharmacology, Tokushima University Graduate School of Biomedical Sciences, Tokushima, Japan
[8] Kondo Naika Hospital, Tokushima, Japan
[9] Anan Medical Center, Tokushima, Japan
[10] Fujii Memorial Institute of Medical Sciences, Tokushima University, Tokushima, Japan

Aims: Thrombin exerts various pathophysiological functions by activating protease-activated receptors (PARs), and thrombin-induced activation of PARs promotes the development of non-alcoholic fatty liver disease (NAFLD). Since heparin cofactor II (HCII) specifically inactivates thrombin action, we hypothesized that plasma HCII activity correlates with the severity of NAFLD.

Methods: A cross-sectional study was conducted. Plasma HCII activity and noninvasive clinical markers of hepatic fibrosis including fibrosis-4 (FIB-4) index, NAFLD fibrosis score (NFS) and aspartate aminotransferase-to-platelet ratio index (APRI) were determined in 305 Japanese patients with type 2 diabetes mellitus (T2DM). The relationships between plasma HCII activity and the clinical markers were statistically evaluated.

Results: Multiple regression analysis including confounding factors showed that plasma HCII activity independently contributed to decreases in FIB-4 index ($p<0.001$), NFS ($p<0.001$) and APRI ($p=0.004$). In addition, logistic regression analysis for the prevalence of advanced hepatic fibrosis defined by the cutoff points of the clinical scores showed that plasma HCII activity was the sole and common negative factor for prevalence of advanced hepatic fibrosis (FIB-4 index: $p=0.002$, NFS: $p=0.026$ and APRI: $p=0.012$).

Conclusions: Plasma HCII activity was inversely associated with clinical hepatic fibrosis indices including FIB-4 index, NFS and APRI and with the prevalence of advanced hepatic fibrosis in patients with T2DM. The results suggest that HCII can serve as a novel biomarker for assessment of hepatic fibrosis of NAFLD in patients with T2DM.

Key words: Thrombin, Protease-activated receptors, Heparin cofactor II, NAFLD, T2DM

Introduction

Non-alcoholic fatty liver disease (NAFLD) includes non-alcoholic fatty liver (NAFL) and non-alcoholic steatohepatitis (NASH), and NASH can progress to cirrhosis and hepatocellular carcinoma.

The prevalence of NAFLD has been increasing worldwide, and it is associated with components of metabolic syndrome including obesity, hyperglycemia, dyslipidemia, and hypertension[1-3]. In addition, it has been reported that NAFLD itself is an independent risk factor for the development of cardiovascular disease (CVD)[4] and that NAFLD is associated with the prognosis of CVD independent of known risk factors[5].

Although liver biopsy is the current gold standard method for staging hepatic fibrosis in patients with NAFLD, the procedure is too invasive for a first-line screening approach. In addition, histological lesions are unevenly distributed throughout the liver tissue and there is critical problem of possible interobserver and intraobserver variability in pathological diagnosis. Therefore, evaluating test accuracy with an imperfect reference standard such as a liver biopsy has the risk of underestimating NASH and hepatic fibrosis severity[6]. On the other hand, many noninvasive clinical scoring systems based on simple clinical or laboratory indices for estimating hepatic fibrosis have been developed. These scoring systems include fibrosis-4 (FIB-4) index[7-9], NAFLD fibrosis score (NFS)[10] and aspartate aminotransferase-to-platelet ratio index (APRI)[11, 12]. While histological fibrosis predicts disease progression, prognostication of NAFLD-related events using such noninvasive markers is an appealing alternative to liver biopsy, and direct comparisons have shown the ability of those noninvasive markers for risk stratification of patients with performance that is comparable to or even better than that of a liver biopsy[13, 14].

Thrombin is a serine protease that plays an important role in the coagulation cascade pathway and it is also related to vascular inflammation through activation of thrombin receptors (known as protease activator receptors: PARs)[15]. Recent investigations have demonstrated that plasma levels of thrombin are increased in patients with obesity and NAFLD[16, 17]. Moreover, Kassel *et al.* showed that PAR-1 is required for liver inflammation and steatosis in mice fed a Western diet[18].

Heparin cofactor II (HCII) is a serine protease inhibitor (i.e., a serpin) with a molecular weight of 65.6 kDa. HCII is synthesized by hepatocytes and secreted into the bloodstream at a concentration of approximately 1.0 μmol/L, and upon activation by binding to dermatan sulfate proteoglycans, it specifically inhibits thrombin activities in various tissue matrices[19]. We and others have shown that HCII exerts a negative impact on the development of CVDs[20-24] and diabetic kidney disease represented by albuminuria[25] and that it ameliorates insulin resistance[26]. Taken together, the results of studies indicate the possibility that HCII has a negative association with hepatic fibrosis of NAFLD through inactivation of thrombin action.

Aim

This study was designed to verify whether there is a correlation between plasma HCII activity and severity of NAFLD classified by noninvasive clinical indices including FIB-4 index, NFS and APRI in patients with type 2 diabetes.

Materials

Study Design, Subjects and Ethics Statement

This study was a cross-sectional study. We consecutively recruited 305 Japanese individuals (177 males and 128 females) who were outpatients or inpatients with type 2 diabetes mellitus (T2DM). The diagnosis of T2DM followed the criteria proposed by the report of the Expert Committee on the Diagnosis and Classification of Diabetes Mellitus[27].

All of the subjects were older than 20 years of age and were recruited consecutively from the Department of Endocrinology and Metabolism of Tokushima University Hospital, the Anan Medical Center, Kondo Naika Hospital, and Minami Municipal National Insurance Hospital (Tokushima, Japan) between July 2017 and December 2020. The exclusion criteria for the patients with diabetes were as follows: 1) patients with advanced cancer, 2) patients with secondary diabetes such as steroid-induced diabetes or pancreatic diabetes, 3) patients who were pregnant, and 4) patients with advanced renal disease and a serum creatine level of >2.0 mg/dL.

All of the subjects enrolled in this study underwent a standardized interview and a physical examination. Current smokers were defined as subjects who had smoked within the past 2 years. Body mass index was calculated as an index of obesity. Blood pressure (BP) was measured twice in each participant in a sitting position using an automatic sphygmomanometer, and the BP values were averaged. Hypertensive patients were defined as those with

Address for correspondence: Ken-ichi Aihara, Department of Community Medicine and Medical Science, Tokushima University Graduate School of Biomedical Sciences, 3-18-15 Kuramoto-cho, Tokushima 770-8503, Japan   E-mail: aihara@tokushima-u.ac.jp

systolic BP (SBP) of ≥ 140 mmHg and/or diastolic BP of ≥ 90 mmHg or those receiving antihypertensive agents. Patients with dyslipidemia were defined as those with a low-density lipoprotein cholesterol (LDL-C) level of ≥ 140 mg/dL, a triglyceride (TG) level of ≥ 150 mg/dL, a high-density lipoprotein cholesterol (HDL-C) level of < 40 mg/dL, or those receiving lipid-lowering agents.

Our study followed the institutional guidelines of each hospital (Tokushima University Hospital, Anan Medical Center, Kondo Naika Hospital, and Minami Municipal National Insurance Hospital) and was approved by each hospital's Institutional Review Board (ethics committees of Tokushima University Hospital (date of approval: July 20, 2020; approval ID 3760), of Anan Medical Center (date of approval: January 17, 2021; approval ID 6), of Kondo Naika Hospital (date of approval: November 25, 2021; approval ID: 2021-12), and of Minami Municipal National Insurance Hospital (date of approval: June December 10, 2021; approval ID: R3-1)). Prior informed consent was obtained from all patients according to the Declaration of Helsinki.

Biochemical Analyses

Blood samples were collected from each patient and used for determining blood cell counts, plasma glucose (PG), HbA1c, and serum biochemical parameters including LDL-C, TG, HDL-C, uric acid (UA), aspartate aminotransferase (AST), alanine aminotransferase (ALT), creatinine (Cr) and albumin (ALB). PG level and serum levels of LDL-C, TG, HDL-C, UA, AST, ALT, Cr and ALB were measured by enzymatic methods. HbA1c level was measured by the latex agglutination assay.

Measurements of Plasma Fibrinogen, Prothrombin Time-International Normalized Ratio (PT-INR) and Plasma HCII Activity

Blood was drawn and collected into a tube containing a 1/10 volume of 3.8% sodium citrate and was centrifuged at 2000×g for 20 minutes. Plasma was stored at −80℃ until use. Plasma fibrinogen concentrations were determined by performing thrombin coagulation tests. Prothrombin time-international normalized ratio (PT-INR) was determined by the coagulation time measurement method. Plasma HCII activities were measured on the basis of antithrombin activities in the presence of dermatan sulfate, which were determined using the Stachrom® HCII Assay Kit (Diagnostica Stago). The intra-assay and inter-assay coefficients of variation of this kit were 3.9% and 4.3%, respectively.

Determination of Severity of NAFLD Represented by FIB-4 Index, NFS and APRI

We used three clinical scoring systems including FIB-4 index, NFS and APRI for estimating hepatic fibrosis. Each index or score for assessment of the severity of NAFLD was determined by the following formulas.

∗FIB-4 index = Age (years) × AST (U/L) / [platelet count (× 10^9 / L) × √ALT (U/L)][7]

∗NFS = -1.675 + 0.037 × age (year) + 0.094 × BMI (kg/m²) + 1.13 × IFG/diabetes (yes = 1, no = 0) + 0.99 × AST / ALT ratio − 0.013 × platelet count (× 10^9/L) − 0.66 × ALB (g/dL)[10]

∗APRI = 100 × AST (U/L) / upper limit of normal AST (U/L) / platelet count (× 10^9/L)[11]

The proposed cutoff points of the clinical scores were as follows. Subjects with FIB-4 index < 1.3 were considered as being at the lowest risk of hepatic fibrosis, subjects with FIB-4 index ≥ 1.3 to 2.67 were considered as having a moderate risk, and subjects with FIB-4 index > 2.67 were classified as having a high risk of advanced hepatic fibrosis[7]. Subjects with NFS < -1.455 were classified as not having advanced hepatic fibrosis, subjects with NFS ≥ -1.455 to < 0.676 were classified as having intermediate hepatic fibrosis, and subjects with NFS ≥ 0.676 were classified as having advanced hepatic fibrosis[10]. Subjects with APRI > 0.5 were defined as having hepatic fibrosis and subjects with APRI > 1.5 were defined as having cirrhosis[11, 12].

Statistical Analysis

Continuous variables were averaged and expressed as means ± standard deviation (SD). Categorical variables were compared by performing the χ^2 test or Fisher's exact test. For comparisons between two groups, we performed the Mann-Whitney U test or Student's t-test for numeric variables depending on the variables' distribution. The degrees of associations between the clinical markers of hepatic fibrosis (FIB-4 index, NFS and APRI) and each clinical variable including plasma HCII activity were determined by performing multivariate regression and logistic regression analyses. To determine optimal variables for the multivariate analyses, we selected general NAFLD risk factors, referring to previous studies[28-30]. In addition, to clarify the HCII contribution in an NAFLD condition, we also included ALB, PT-INR, and fibrinogen, which are useful variables to reflect hepatic synthesis capacity.

The analyses were performed by using GraphPad Prism 8 (GraphPad Software, San Diego, CA). A cutoff value for the detection of advanced hepatic fibrosis was determined by receiver operating characteristic (ROC) analysis using EZR (Saitama

Medical Center, Jichi Medical University). The threshold for statistical significance was set at $p < 0.05$.

Results

Characteristics of the Subjects

The physical and laboratory-determined characteristics of subjects enrolled in this study are shown in **Table 1**. On average, the females enrolled in this study had higher HDL-C levels than those in the males. The male patients showed higher casual PG levels and higher serum levels of UA and Cr than those in the female patients. No significant gender differences were observed in other biochemical and hemostatic factors including plasma HCII activity. The percentages of male patients who were current smokers and α glucosidase inhibitor users were significantly higher than the percentages of female patients. A greater percentage of female patients were statin users. The oral antidiabetic drug prescribing pattern in this study and the current prescribing situation in Japan[31] were very similar.

Prevalence of NAFLD Defined by Noninvasive Clinical Scores in Participants

Approximately half of the subjects had a FIB-4 index greater than 1.3 and were presumed to have significant fibrosis. More than 60% of the subjects had NFS greater than -1.455 and were defined as having intermediate to advanced hepatic fibrosis. Approximately 10% of the subjects had APRI greater than 0.5 and were diagnosed as having hepatic fibrosis **(Supplemental Fig. 1)**.

Associations between Plasma HCII Activity and NAFLD Clinical Scores

Scatter plots between plasma HCII activity and each NAFLD clinical score are shown in **Fig. 1**. Simple linear regression analysis showed that plasma HCII activity correlated negatively with FIB-4 index ($p < 0.001$), NFS ($p < 0.001$) and APRI ($p = 0.004$). Multiple regression analysis showed that plasma HCII activity was found to be the sole negative factor against increments of these clinical scoring systems of NAFLD (FIB-4 index: $p < 0.001$, NFS: $p < 0.001$, APRI: $p = 0.005$) **(Table 2)**. Since it is well known that pharmacological interventions influence the severity of NAFLD in patients with diabetes, we next performed multiple regression analysis with the confirmed independent variables shown in **Table 2** and the medications used in this study. Conclusively, plasma HCII activity remained a significant sole common negative factor against increment of the three NAFLD clinical scores regardless of the medications taken in the present study (FIB-4 index: $p < 0.001$, NFS: $p < 0.001$, APRI: $p = 0.004$) **(Table 3)**.

In addition, we evaluated the associations of plasma HCII activity with configuration factors of the formula for each clinical index by simple linear regression analysis and we found that plasma HCII activity had significant associations with age, BMI, platelet count, ALB, ALT, AST-to-ALT ratio and √ALT but not with AST **(Supplemental Fig. 2)**. However, multiple linear regression analysis showed no independent association of plasma HCII activity with AST or ALT **(Supplemental Table 1)**. Since it was reported that only a small percentage of patients with steatosis or liver fibrosis had elevated levels of AST and ALT and since AST or ALT alone has been recognized as insufficient initial screening of NAFLD[12], we speculate that HCII reflected the NAFLD clinical indices mainly via associations with age, platelet count and ALB but not with hepatic transaminases. Furthermore, the nonuniform relationships between plasma HCII activity and the clinical indices seemed to be related to formula differences in weighting of AST.

Association between Plasma HCII Activity and Prevalence of Advanced Hepatic Fibrosis

Next, we performed multiple logistic regression analysis to determine significant clinical factors for the detection of advanced hepatic fibrosis. The cutoff points we used were FIB-4 index greater than 2.67, NFS greater than 0.676 and APRI greater than 0.5, which indicate cirrhosis[7], advanced hepatic fibrosis[10] and hepatic fibrosis[11], respectively. As shown in **Fig. 2**, HCII was an independent and sole common negative factor for advanced hepatic fibrosis defined by the three indices (FIB-4 index: increase in odds ratio with plasma HCII at 1% activity to 0.946 (increase in odds ratio with HCII at 10% activity to 0.574), $p = 0.002$; NFS: increase in odds ratio with plasma HCII at 1% activity to 0.971 (increase in odds ratio with HCII at 10% activity to 0.745), $p = 0.026$; APRI: increase in odds ratio with plasma HCII at 1% activity to 0.971 (increase in odds ratio with HCII at 10% to 0.745), $p = 0.012$). In addition, we performed ROC analysis to determine the optimal cutoff point for the detection of advanced hepatic fibrosis in each clinical index and found that the optimal cutoff points were ≤ 86.6% for FIB-4 index, ≤ 85.3% for NFS and ≤ 87.8% for APRI **(Supplemental Fig. 3)**.

Discussion

Previous studies have demonstrated that an aberrant coagulation state is closely associated with the

Table 1. Clinical Characteristics of the Subjects Enrolled in the Cross-Sectional Study

	Total	Male	Female	*p* value (Male vs Female)
Number of Subjects	305	177	128	
Age (years)	66.5 ± 10.9	65.1 ± 11.2	68.3 ± 10.1	0.026
BMI (kg/m²)	25.6 ± 4.8	25.4 ± 4.7	25.9 ± 5.0	0.324
SBP (mmHg)	131.2 ± 16.6	131.6 ± 16.8	130.5 ± 16.2	0.625
LDL-C (mg/dL)	100.2 ± 31.0	97.8 ± 31.0	103.6 ± 30.6	0.392
TG (mg/dL)	138.6 ± 77.7	143.0 ± 83.3	132.4 ± 68.7	0.294
HDL-C (mg/dL)	54.7 ± 14.1	52.3 ± 14.5	58.1 ± 12.8	<0.001
Casual PG (mg/dL)	151.7 ± 52.9	160.0 ± 57.1	140.4 ± 43.9	0.001
HbA1c (%)	6.9 ± 1.1	7.0 ± 1.2	6.8 ± 0.9	0.298
UA (mg/dL)	5.2 ± 1.2	5.4 ± 1.2	4.8 ± 1.2	<0.001
Cr (mg/dL)	0.80 ± 0.20	0.86 ± 0.22	0.68 ± 0.19	<0.001
eGFR (ml/min/1.73m²)	73.4 ± 20.2	74.4 ± 20.9	72.1 ± 19.0	0.624
ALB (g/dL)	4.20 ± 0.30	4.20 ± 0.36	4.16 ± 0.33	0.217
Plasma Fibrinogen (mg/dL)	338.9 ± 76.9	339.7 ± 78.0	337.9 ± 75.3	0.813
PT-INR	1.18 ± 0.21	1.18 ± 0.19	1.19 ± 0.23	0.790
Plasma HCII Activity (%))	94.4 ± 17.9	94.0 ± 18.1	95.0 ± 17.5	0.472
AST (U/L)	24.9 ± 12.1	25.3 ± 13.3	24.2 ± 10.0	0.725
ALT (U/L)	26.3 ± 20.4	27.2 ± 22.5	25.1 ± 16.9	0.980
Platelets (10⁹/L)	221.8 ± 56.4	216.9 ± 53.4	228.6 ± 59.5	0.090
FIB-4 index	1.64 ± 0.75	1.67 ± 0.83	1.60 ± 0.61	0.864
NFS	-0.24 ± 1.13	-0.24 ± 1.16	-0.23 ± 1.08	0.684
APRI	0.34 ± 0.18	0.36 ± 0.19	0.32 ± 0.16	0.264
Current Smoking (*n*, (%))	52 (17.0)	48 (27.1)	4 (3.1)	<0.001
Hypertension (*n*, (%))	215 (70.5)	119 (67.2)	96 (75.0)	0.142
Dyslipidemia (*n*, (%))	217 (71.1)	118 (66.7)	99 (77.3)	0.042
Duration of Diabetes (years)	10.5 ± 8.1	11.0 ± 8.6	9.8 ± 7.4	0.362
ARB or ACEi (*n*, (%))	150 (49.2)	78 (44.1)	72 (56.3)	0.036
Ca blocker (*n*, (%))	130 (42.6)	70 (39.5)	60 (46.9)	0.202
β blocker (*n*, (%))	22 (7.2)	9 (5.1)	13 (10.2)	0.068
MR antagonist (*n*, (%))	7 (2.3)	3 (1.7)	4 (3.1)	0.411
Statin (*n*, (%))	147 (48.2)	71 (40.1)	76 (59.4)	0.001
Ezetimibe (*n*, (%))	19 (6.2)	8 (4.5)	11 (8.6)	0.146
Other Hypolipidemic Agents (*n*, (%))	16 (5.2)	12 (6.8)	4 (3.1)	0.158
Antiplatelet (*n*, (%))	36 (11.8)	25 (14.1)	11(8.6)	0.089
SU or Glinide (*n*, (%))	59 (19.3)	33 (18.6)	26 (20.3)	0.716
Metformin (*n*, (%))	150 (49.2)	85 (48.0)	65 (50.8)	0.634
DPP-4i (*n*, (%))	206 (67.5)	119 (67.2)	87 (68.0)	0.892
SGLT2i (*n*, (%))	68 (22.3)	44 (24.9)	24 (18.8)	0.206
αGI (*n*, (%))	66 (21.6)	45 (25.4)	21 (16.4)	0.059
Pioglitazone (*n*, (%))	21 (6.9)	14 (7.9)	7 (5.5)	0.406
Insulin (*n*, (%))	52 (17.0)	34 (19.2)	18 (14.1)	0.238
GLP-1RA (*n*, (%))	28 (9.2)	15 (8.5)	13 (10.2)	0.616

The values are presented as mean ± SD or *n* (%).
Abbreviations: ACEi; angiotensin-converting enzyme inhibitor, ALB; albumin, APRI; aspartate aminotransferase-to-platelet ratio index, ARB; angiotensin II receptor blocker, BMI; body mass index, Cr; creatinine, DPP-4i; dipeptidyl peptidase-4 inhibitor, eGFR; estimated glomerular filtration rate, FIB-4; fibrosis-4, GLP-1RA; glucagon-like peptide-1 receptor agonist, HbA1c; hemoglobin A1c, HDL-C; high-density lipoprotein cholesterol, LDL-C; low-density lipoprotein cholesterol, MR; mineral corticoid receptor, NFS; NAFLD fibrosis score, PG; plasma glucose; PT-INR; prothrombin time-international normalized ratio, SBP; systolic blood pressure, SGLT2i; sodium-glucose cotransporter 2 inhibitor, SU; sulfonyl urea, TG; triglyceride, αGI; alpha-glucosidase inhibitor
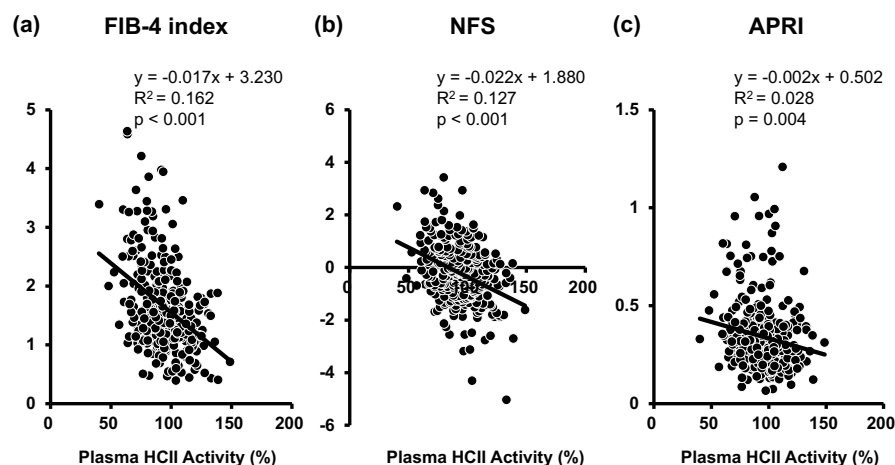
Fig. 1. Scatter plots between plasma HCII activity and NAFLD clinical scores in patients with T2DM

(a) Scatter plot between plasma HCII activity and FIB-4 index.
(b) Scatter plot between plasma HCII activity and NFS.
(c) Scatter plot between plasma HCII activity and APRI.
Data for 177 males and 128 females in this study are included in the scatter plots.

Table 2. Multiple Regression Analysis for Determinants of NAFLD Clinical Scores

Variables	FIB-4 index		NFS		APRI	
	t value	*p* value	t value	*p* value	t value	*p* value
Age	9.65	<0.001	11.77	<0.001	1.89	0.060
Male	2.70	0.007	1.87	0.063	2.47	0.014
BMI	1.30	0.195	8.89	<0.001	2.89	0.004
SBP	-0.27	0.785	0.07	0.942	-0.72	0.474
LDL-C	-1.29	0.198	-2.42	0.016	-0.02	0.984
TG	1.18	0.238	-0.11	0.913	1.55	0.122
HDL-C	4.04	<0.001	3.87	0.001	1.77	0.078
HbA1c	-1.69	0.092	-1.8	0.074	-0.07	0.947
UA	1.68	0.094	1.28	0.203	1.56	0.119
Cr	1.10	0.272	2.11	0.036	-0.86	0.389
ALB	-1.96	0.051	-4.2	<0.001	-0.78	0.435
Plasma Fibrinogen	0.27	0.789	0.33	0.742	-0.83	0.409
PT-INR	0.90	0.371	1.29	0.199	0.19	0.846
Plasma HCII Activity	-4.71	<0.001	-4.62	<0.001	-2.84	0.005
Current Smoking	-1.01	0.314	-0.05	0.961	-1.23	0.220
Hypertension	-2.00	0.047	-1.94	0.054	-0.65	0.519
Dyslipidemia	1.63	0.103	1.34	0.183	2.02	0.044
Duration of Diabetes	-1.62	0.107	-0.54	0.593	-1.46	0.146

development of NAFLD [17, 32] and that patients with metabolic syndrome generate greater amounts of thrombin than do patients without metabolic syndrome [33]. Kopec *et al.* reported that mice fed a high-fat diet (HFD) showed hepatic inflammation with increased macrophage accumulation, increased hepatic fibrin deposition, and increasing plasma thrombin-antithrombin III complex levels, a stable marker of thrombin generation. In addition, treatment with dabigatran, which is a synthetic direct thrombin inhibitor, significantly reduced hepatic inflammation, hepatic fibrin deposition, hepatocellular injury, and steatosis in mice fed an HFD [34]. These findings are consistent with the notion that thrombin activation plays a pivotal role in the development of NAFLD. Kassel *et al.* reported that monocyte chemotactic

Table 3. Multiple Regression Analysis Including Identified Variables and Medications Used for Determinants of NAFLD Clinical Scores

Variables	FIB-4 index		NFS		APRI	
	t value	*p* value	t value	*p* value	t value	*p* value
Age	8.98	<0.001	11.91	<0.001	1.04	0.300
Male	3.45	0.001	3.29	0.001	2.30	0.022
BMI	1.67	0.097	8.63	<0.001	3.60	0.001
HDL-C	3.99	<0.001	3.31	0.001	1.46	0.147
LDL-C	-0.59	0.556	-1.15	0.250	0.43	0.664
ALB	-1.85	0.065	-4.44	<0.001	-0.66	0.507
Hypertension	-1.04	0.300	-0.87	0.384	-0.75	0.452
Plasma HCII Activity	-4.97	<0.001	-4.92	<0.001	-2.91	0.004
ARB or ACEi	0.23	0.819	0.10	0.924	0.30	0.766
Ca Blocker	-0.98	0.327	-0.64	0.522	-0.80	0.424
β blocker	0.12	0.902	0.03	0.976	-1.56	0.121
MR Antagonist	-1.13	0.261	-1.17	0.242	-0.87	0.383
Statin	1.58	0.116	2.34	0.020	1.68	0.094
Ezetimibe	1.20	0.232	-0.25	0.801	1.86	0.064
Other Hypolipidemic Agents	0.19	0.847	-1.11	0.269	1.34	0.182
Antiplatelet	1.61	0.108	0.61	0.540	0.97	0.332
SU or Glinide	-0.87	0.384	-2.33	0.021	0.49	0.622
Metformin	-0.02	0.984	0.32	0.749	-0.65	0.520
DPP-4i	1.73	0.086	0.98	0.330	0.16	0.877
SGLT2i	-0.82	0.411	-0.42	0.673	-0.41	0.683
αGI	1.25	0.212	0.29	0.775	-1.38	0.170
Pioglitazone	0.25	0.806	0.02	0.988	0.15	0.884
Insulin	-0.06	0.954	1.38	0.168	-1.61	0.108
GLP-1RA	0.72	0.474	0.47	0.641	0.25	0.801

protein 1 (MCP-1) plays a role in the development of hepatic steatosis and insulin resistance by causing infiltration of macrophages into adipose tissue or the liver[18]. In our previous study, we found that MCP-1 gene expression in 3T3-L1 adipocytes is markedly increased by thrombin treatment[35]. These observations suggest that thrombin-PAR system activation is associated with increased adiposity and enhanced expression of MCP-1 in the liver.

We generated heterozygous HCII-deficient mice and demonstrated that the mutant mice manifested exaggerated cardiovascular remodeling[22, 36-38] and enhanced insulin resistance with increased gluconeogenesis[26]. In addition, we previously found that cuff injury and HFD feeding prominently augmented MCP-1 gene expression in arterial walls and in epidydimal fat of heterozygous HCII-deficient mice, respectively[22, 26]. Taken together, the results indicate the possibility that HCII is negatively involved in the pathogenesis of NAFLD via inactivation of the thrombin-MCP-1 axis.

Limitations

A limitation of this study is that the results cannot be extended to the general population because (i) we enrolled only patients with T2DM and (ii) we previously found that subjects without cardiovascular risk factors (including diabetes) had higher plasma HCII activity levels than those in subjects with one or more cardiovascular risk factors[21, 26]. Furthermore, because this study did not include imaging examinations to evaluate fibrosis and stiffness of the liver such as elastography using ultrasound or MRI, association analysis between plasma HCII activity and severity of liver stiffness evaluated by those noninvasive imaging devices would corroborate our hypothesis. Finally, since this cross-sectional study is not able to clarify the causal relationship between plasma HCII activity and the development of NAFLD, additional large-scale and longitudinal analysis is essential to clarify this clinical question.
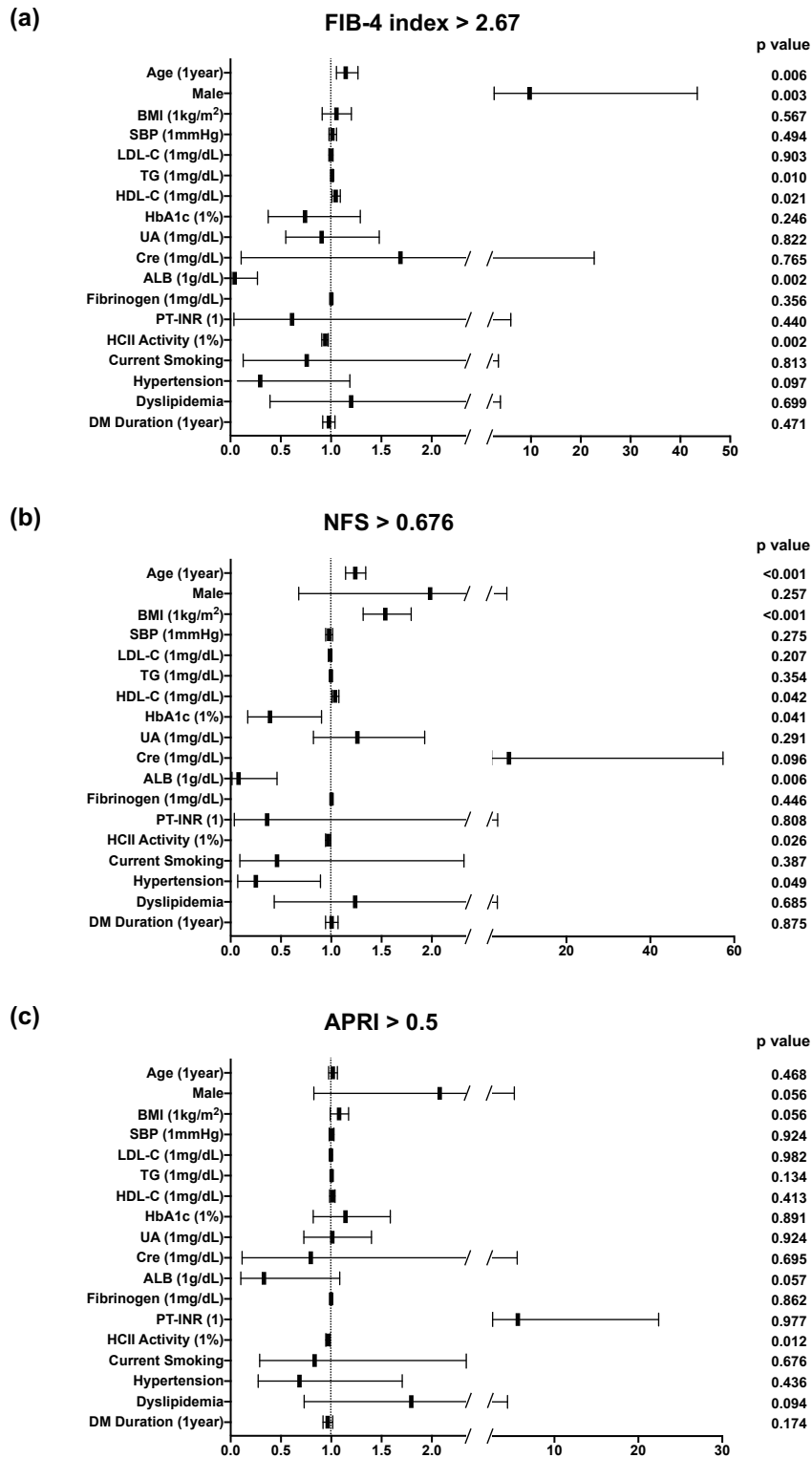
Fig. 2. Forest plots for prevalence of advanced hepatic fibrosis defined by NAFLD clinical scores in patients with T2DM

(a) Forest plot for prevalence of advanced hepatic fibrosis defined as FIB-4 index >2.67.
(b) Forest plot for prevalence of advanced hepatic fibrosis defined as NFS >0.676.
(c) Forest plot for prevalence of advanced hepatic fibrosis defined as APRI >0.5.
Data for 177 males and 128 females in this study are included in the forest plots.

Conclusion

The present study demonstrated that plasma HCII activity has an inverse association with the severity of NAFLD in patients with T2DM. In diabetic patients with low plasma HCII activity, early comprehensive intervention including lifestyle modifications and available pharmacological treatment might be needed for patients with T2DM who are suffering from NAFLD.

Acknowledgements

Conflicts of Interest

Funding

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(a) FIB-4 index

< 1.3 : normal
1.3-2.67 : hepatic fibrosis
> 2.67 : cirrhosis
(Clin Gastroenterol Hepatol 2009; 7: 1104-1112)

(b) NFS

< -1.455 : normal
-1.455-0.676 : intermediate hepatic fibrosis
> 0.676 : advanced hepatic fibrosis
(Hepatology. 2007; 45: 846-54)

(c) APRI

> 0.5 : hepatic fibrosis
> 1.5 : cirrhosis
(Hepatology. 2003; 38: 518-26.)
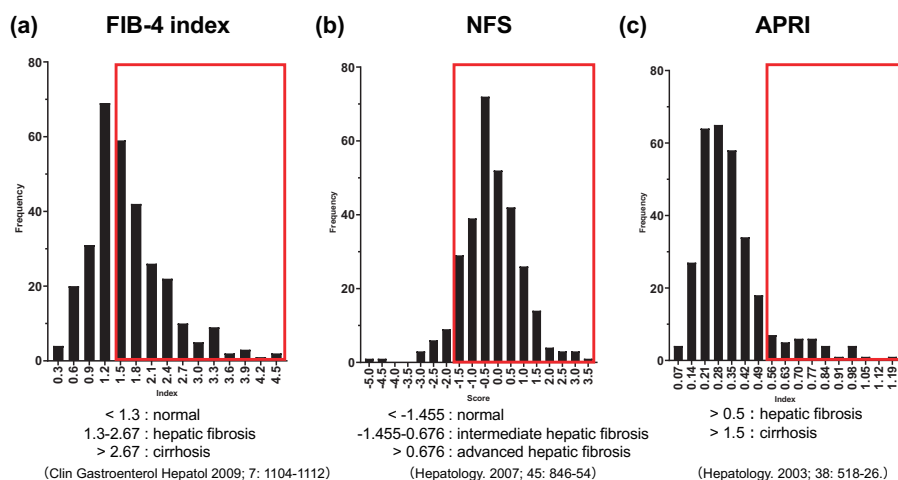
Supplemental Fig. 1. Histogram of the subjects in each NAFLD clinical score

(a) FIB-4 index, (b) NFS, (c) APRI
Red-colored frame indicates subjects defined as NAFLD in each clinical scoring system.

Supplemental Table1. Multiple Regression Analysis for Determinants of AST and ALT

Variables	AST		ALT	
	t value	p value	t value	p value
Age	0.97	0.330	-1.36	0.174
Male	1.98	0.049	1.34	0.181
BMI	3.23	0.001	4.22	<0.001
SBP	-0.84	0.400	-0.94	0.350
LDL-C	1.00	0.316	1.29	0.197
TG	2.01	0.046	1.93	0.054
HDL-C	1.23	0.220	-1.12	0.266
HbA1c	0.48	0.634	2.11	0.036
UA	1.83	0.069	1.52	0.129
Cr	-1.69	0.093	-1.68	0.095
ALB	-0.53	0.599	0.31	0.758
Plasma Fibrinogen	-0.72	0.471	-1.28	0.202
PT-INR	-0.18	0.858	-0.37	0.709
Plasma HCII Activity	-0.86	0.388	-0.27	0.785
Current Smoking	-1.50	0.135	-1.61	0.108
Hypertension	0.07	0.945	0.34	0.738
Dyslipidemia	0.97	0.331	0.66	0.512
Duration of Diabetes	-0.87	0.387	-0.33	0.744
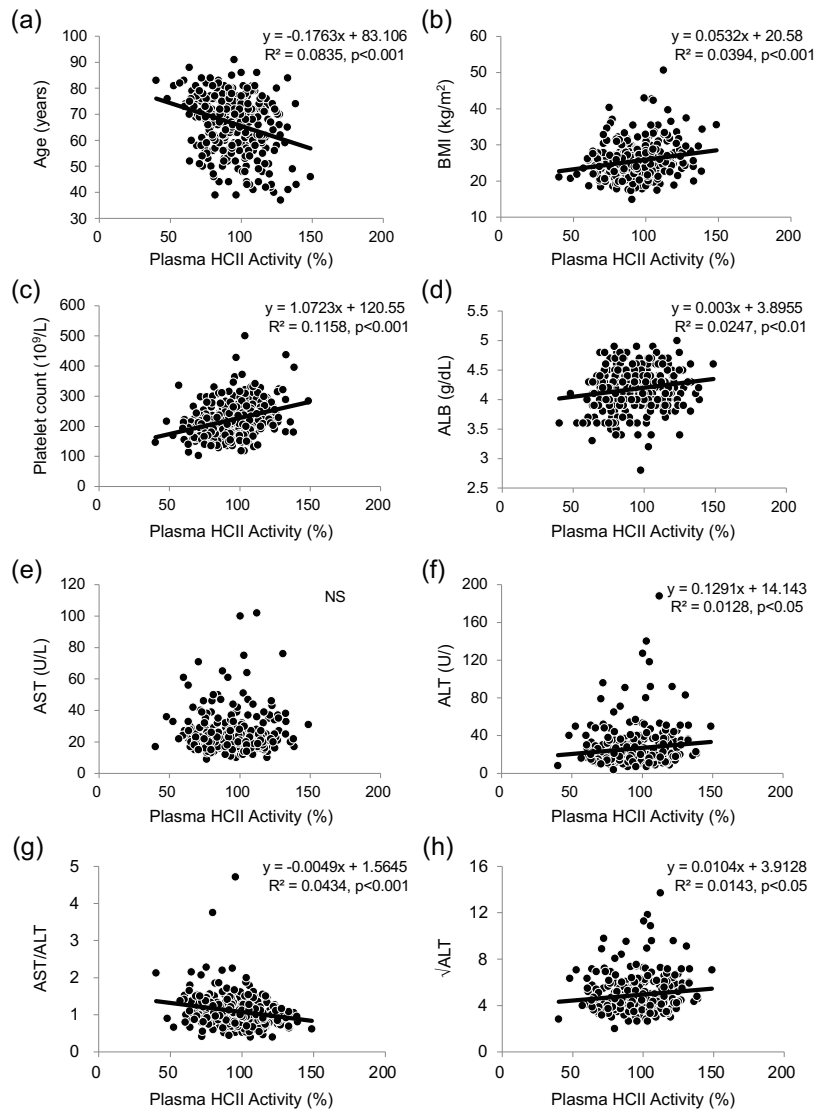
Supplemental Fig. 2. Scatter plots between plasma HCII activity and configuration factors of the formula for each NAFLD clinical score

(a) Age, (b) BMI, (c) Platelet count, (d) ALB, (e) AST, (f) ALT, (g) AST/ALT, (h) √ALT

(a)

(b)

(c)
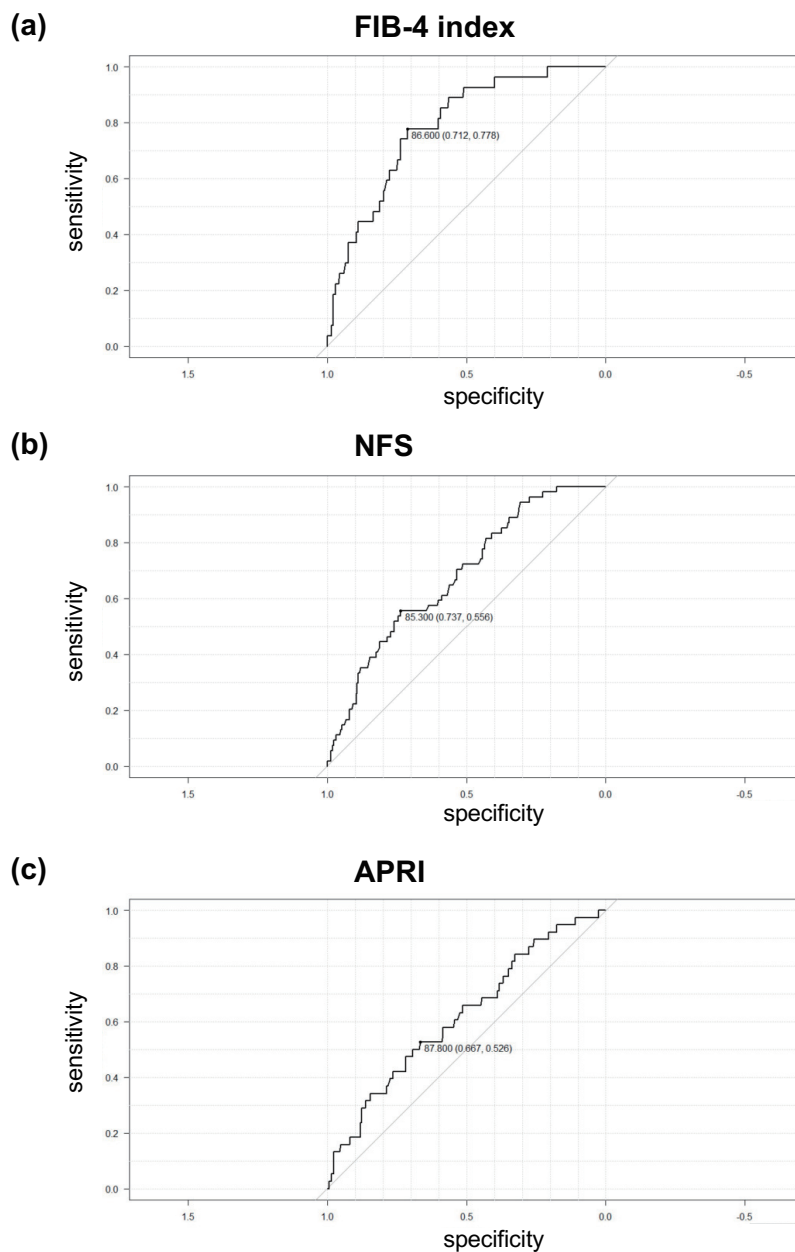
Supplemental Fig. 3. ROC curve analysis of hepatic fibrosis in each NAFLD clinical score
(a) FIB-4 index, (b) NFS, (c) APRI