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ORIGINAL ARTICLE

Plasma heparin cofactor II activity is inversely associated with albuminuria and its annual deterioration in patients with diabetes

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ABSTRACT

Aims/Introduction: Thrombin exerts various pathophysiological functions by activating protease-activated receptors (PARs). Recent data have shown that PARs influence the development of glomerular diseases including diabetic kidney disease (DKD) by regulating inflammation. Heparin cofactor II (HCII) specifically inactivates thrombin; thus, we hypothesized that low plasma HCII activity correlates with DKD development, as represented by albuminuria.

Materials and Methods: Plasma HCII activity and spot urine biomarkers, including albumin and liver-type fatty acid-binding protein (L-FABP), were determined as the urine albumin-to-creatinine ratio (uACR) and the urine L-FABP-to-creatinine ratio (uL-FABPCR) in 310 Japanese patients with diabetes mellitus (176 males and 134 females). The relationships between plasma HCII activities and those DKD urine biomarkers were statistically evaluated. In addition, the relationship between plasma HCII activities and annual uACR changes was statistically evaluated for 201/310 patients (115 males and 86 females).

Results: The mean plasma HCII activity of all participants was 93.8 ± 17.7%. Multivariate-regression analysis including confounding factors showed that plasma HCII activity independently contributed to the suppression of the uACR and log-transformed uACR values ($P = 0.036$ and $P = 0.006$, respectively) but not uL-FABPCR ($P = 0.541$). In addition, plasma HCII activity significantly and inversely correlated with annual uACR and log-transformed uACR increments after adjusting for confounding factors ($P = 0.001$ and $P = 0.014$, respectively).

Conclusions: The plasma HCII activity was inversely and specifically associated with glomerular injury in patients with diabetes. The results suggest that HCII can serve as a novel predictive factor for early-stage DKD development, as represented by albuminuria.

INTRODUCTION

Diabetic kidney disease (DKD) is an important microvascular complication of diabetes and the most common primary disease in dialysis patients in Japan (38.8% of patients) [1]. DKD prevention and early diagnosis of disease onset and progression are pivotal for maintaining a good prognosis and quality of life for diabetic patients and viable medical economics.

The natural history of DKD includes glomerular hyperfiltration, progressive albuminuria, a declining estimated glomerular-filtration rate (eGFR), and progression to end-stage renal disease. The presence of albuminuria implies dysfunction of the

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glomerular filtration barrier due to persistent hyperglycemia, hypertension, lipid abnormalities, or a combination of these pathological conditions. In addition, albuminuria has been shown to be a prognostic marker for chronic kidney disease (CKD) and a predictor of cardiovascular mortality [2-6].

Protease-activated receptors (PARs) are members of a family of transmembrane G protein-coupled receptors. Previous findings showed that there are four subtypes of PARs in mammalian cells, that PAR-1 is the most abundant receptor, and that PAR-1 is ubiquitously expressed in human renal cells including glomerular endothelial cells, mesangial cells, and tubular epithelial cells [7]. PARs are activated by endogenous serine proteases (such as thrombin) and the activation of PARs can promote the development of glomerular diseases by enhancing inflammation [8]. Additional data showed that inactivating PARs and/or deleting PAR genes ameliorated glomerular diseases [9-11].

In terms of inhibiting thrombin-mediated PAR activation, we have focused on heparin cofactor II (HCII), which 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 [12]. We and others have shown that HCII prevents the development of cardiovascular diseases (CVDs) [13-20] and ameliorates insulin resistance [21] by attenuating thrombin-mediated PAR activation.

Taken together, the results of previous studies indicate the possibility that thrombin inactivation by HCII would prevent progression of renal diseases such as DKD. In this study, we aimed to clarify the relationship between plasma HCII activity and the development of DKD, as represented by albuminuria in patients with diabetes.

METHODS

Study design, subjects and ethics statement

We consecutively recruited 310 Japanese individuals (176 males and 134 females) who were outpatients or inpatients with type-1 diabetes mellitus or type-2 diabetes mellitus. All subjects were older than 20 years of age and were recruited consecutively from the Department of Endocrinology and Metabolism (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: (i) patient with advanced cancer, (ii) patients with secondary diabetes, such as steroid-induced diabetes or pancreatic diabetes, (iii) patients who were pregnant, (iv) patients with malnutrition including liver cirrhosis, and (v) patients with advanced renal disease and a serum creatine level of >176.8 μmol/L (2 mg/dL). We collected clinical data and urine samples at baseline and 1 year later to perform cross-sectional and longitudinal analyses. The study design is shown in Figure 1.

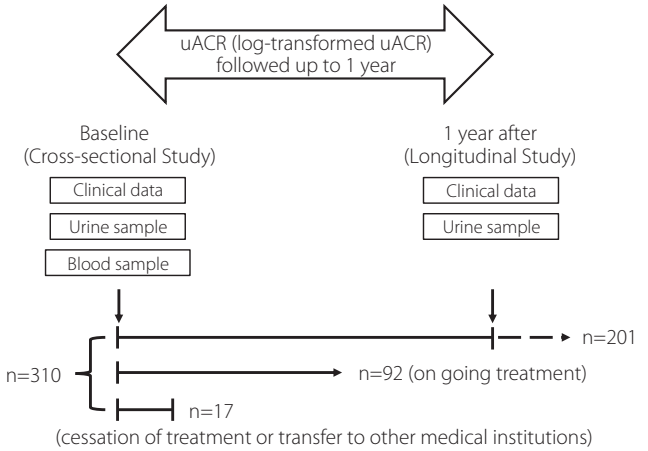

Figure 1 | Schematic representation of the study protocol.

All 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. The body–mass index was calculated as an index of obesity. The blood pressure (BP) of each participant was measured twice in a sitting position, using an automatic sphygmomanometer, and the BP values were averaged. Hypertensive patients were defined as those with a systolic BP (SBP) of ≥140 mm Hg, and/or a diastolic BP of ≥90 mm Hg, or those receiving antihypertensive agents. Patients with dyslipidemia were defined as those with a low-density lipoprotein cholesterol (LDL-C) level of ≥3.63 mmol/L (140 mg/dL), a triglyceride (TG) level of ≥1.70 mmol/L (150 mg/dL), a high-density lipoprotein cholesterol (HDL-C) level of <1.04 mmol/L (40 mg/dL), or those receiving lipid-lowering agents.

Diabetic patients were defined as individuals who were receiving hypoglycemic agents or individuals with a glycosylated hemoglobin A1c (HbA1c) level of ≥47.53 mmol/mol (6.5%).

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 committee of Tokushima University Hospital (date of approval: 28 August 2017; approval ID 2964) of Anan Medical Center (date of approval: 21 July 2017; approval ID 53), of Kondo Naika Hospital (date of approval: 28 September 2017; approval ID: 2017-1, of Minami Municipal National Insurance Hospital (date of approval: 29 June 2020; approval ID: R2-1)). Prior informed consent was obtained from all patients, according to the Declaration of Helsinki.

Biochemical analyses

Blood and single spot urine 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), serum creatinine, the urine albumin-to-creatinine ratio (uACR), and the urine liver-type fatty acid-binding protein (L-FABP)-to-creatinine ratio (uL-FABPCR). The PG and serum levels of LDL-C, TG, HDL-C, UA, and creatinine were measured by enzymatic methods. HbA1c was assayed by performing latex-agglutination assays. Urine albumin levels were assayed by performing turbidimetric immunoassays, and uL-FABP levels were assayed by performing chemiluminescent enzyme immunoassays. eGFR was calculated according to the following formula from the Japanese Society of Nephrology: eGFR (mL/[min·1.73 m^2]) = 194 × serum and creatinine level$^{-1.094}$ × age$^{-0.287}$ (× 0.739, if female) [22].

Measurements of plasma fibrinogen and plasma HCII activities

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 min. Plasma was stored at −80°C until use. Plasma fibrinogen concentrations were determined by performing thrombin-coagulation tests. Plasma HCII activities were measured based on antithrombin activities in the presence of dermatan sulfate, which were determined using the Stachrom® HCII Assay Kit (Diagnostica Stago, Allée Thérésa, Asnières-sur-Seine, France). The intra-assay and inter-assay coefficients of variation of this kit were 3.9% and 4.3%, respectively.

Statistical analysis

Continuous variables with a normal distribution were averaged and expressed as the mean ± standard deviation (SD), and those with a non-normal distribution were expressed as the median (quartile 1 [Q1], Q3). Categorical parameters were expressed as percentages and numbers. Male gender and the presence of hypertension, dyslipidemia, and a current smoking status were coded as dummy variables.

The degree of association between urinary biomarkers (uACR, log-transformed uACR, and uL-FABPCR) and each variable including sex, age, BMI, SBP, serum lipid parameters, UA, creatinine, HbA1c, plasma fibrinogen, plasma HCII activities, history of current smoking, hypertension, and dyslipidemia was determined by performing multiple-regression analyses. Cross-sectional analysis was performed at baseline to determine whether plasma HCII activities were associated with the uACR, log-transformed uACR, and uL-FABPCR levels (n = 310). In addition, longitudinal analysis was performed to determine whether plasma HCII activities were associated with the annual change of uACR (⊿uACR), i.e., the uACR (log-transformed uACR) at 1 year after collecting the baseline data − the uACR (log-transformed uACR) determined at baseline (n = 201; Figure 1).

These analyses were performed using Microsoft Office Excel 2019 (Microsoft, Richmond, CA), GraphPad Prism 8 (GraphPad Software, San Diego, CA, USA), and JMP (SAS Institute Japan Ltd., Tokyo, Japan). The threshold for statistical significance was set at $P < 0.05$.

RESULTS

Baseline characteristics

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 the males. The male patients showed higher casual PG levels and higher serum levels of UA and creatinine than the female patients. No significant gender differences were observed in terms of the BMI, SBP, LDL-C, TG, HbA1c, and plasma HCII activities. The percentage of male patients who were current smokers was significantly higher than that of female patients. A greater percentage of female patients were statin users. The plasma HCII activities declined with age (Figure 2a), as we previously reported [14].

Association between plasma HCII activities and plasma fibrinogen levels

Plasma fibrinogen is a precursor molecule that is converted to fibrin by thrombin and that has been positively associated with the increment of albuminuria [23]. Hence, we estimated the relationship between the plasma plasminogen levels and plasma HCII activities. We found that plasma HCII activities were significantly and positively correlated with plasma fibrinogen levels (R^2 = 0.035, $P < 0.001$; Figure 2b).

Associations between the uACR, log-transformed uACR, and uL-FABPCR values

The uACR level significantly and positively correlated with the uL-FABPCR level (R^2 = 0.280, $P < 0.001$, as shown in Figure 3a. The log-transformed uACR also showed a significant and positive correlation with the uL-FABPCR (R^2 = 0.280, $P < 0.001$), as shown in Figure 3b.

Associations between the plasma HCII activity and the uACR, log-transformed uACR, and uL-FABPCR values

As shown in Figure 4a, b, simple linear-regression analysis for the entire series of subjects showed that plasma HCII activities correlated negatively with the uACR values (R^2 = 0.0099, P = 0.044) and the log-transformed uACR values (R^2 = 0.0156, P = 0.028. In contrast, the plasma HCII activities did not show a significant relationship with the uL-FABPCR values (Figure 4c).

Next, we subjected the univariate baseline parameters to multiple-regression analysis (Table 2). The common risk factors identified for increments of uACR and log-transformed uACR were female gender, SBP, HbA1c, and serum creatinine. Conversely, the plasma HCII activity was found to be the sole protective factor against increments of uACR and log-transformed uACR. Moreover, the independent and inverse correlation of plasma HCII activities with uACR was found even in the patients with normal-ranged albuminuria (<3 mg/mmol (<30 mg/gCr)) (Table S1). Multiple-regression analysis showed that female gender, BMI, SBP, HbA1c and serum creatinine were positive contributors to an increase in the uL-FABPCR, but no relationship was found between the plasma HCII

Table 1 | Clinical characteristics of the subjects enrolled in the cross-sectional study

	Total	Male	Female	*P* value (male vs female)
Number of Subjects	310	176	134	
Type 1 diabetes mellitus	14	6	8	0.282
Age (years; mean [SD])	66.0 ± 11.3	65.0 ± 11.3	67.4 ± 11.2	0.068
BMI (m2/kg; mean [SD])	25.4 ± 4.8	25.3 ± 4.7	25.7 ± 5.0	0.410
SBP (mmHg; mean [SD])	131.2 ± 16.6	131.8 ± 17.1	130.5 ± 16.0	0.510
LDL-C (mmol/L; mean [SD])	2.57 ± 0.78	2.51 ± 0.79	2.64 ± 0.76	0.142
TG (mmol/L; mean [SD])	1.53 ± 0.86	1.58 ± 0.93	1.46 ± 0.74	0.197
HDL-C (mmol/L; mean [SD])	1.43 ± 0.37	1.37 ± 0.39	1.52 ± 0.34	<0.0001
Casual PG (mmol/L; mean [SD])	8.33 ± 2.87	8.74 ± 3.11	7.80 ± 2.41	0.004
HbA1c (mmol/mol; mean [SD])	52 ± 11	52 ± 13	51 ± 8	0.169
HbA1c (%; mean [SD])	6.9 ± 1.0	6.9 ± 1.2	6.8 ± 0.8	0.169
UA (mmol/L; mean [SD])	0.31 ± 0.07	0.33 ± 0.07	0.28 ± 0.07	<0.0001
Cr (μmol/L; mean [SD])	59.4 ± 17.1	65.6 ± 16.5	51.3 ± 14.3	<0.0001
eGFR (ml/min/1.73 m^2)	73.5 ± 20.0	74.2 ± 20.7	72.6 ± 19.0	0.484
uACR (mg/mmol; median [IQR])	1.80 (0.84-6.11)	1.63 (0.76-6.12)	2.0 (0.97-6.0)	0.915
Log-transformed-uACR (mean [SD])	0.41 ± 0.63	0.39 ± 0.64	0.44 ± 0.62	0.455
uL-FABPCR (μg/gCr; median [IQR])	2.51 (1.57-4.62)	2.40 (1.48-4.69)	2.60 (1.60-4.60)	0.541
Plasma Fibrinogen (g/L; mean [SD])	3.39 ± 0.77	3.39 ± 0.79	3.38 ± 0.75	0.980
Plasma HCII Activity (%))	93.8 ± 17.7	93.4 ± 17.9	94.2 ± 17.5	0.509
Current Smoking (*n*, (%))	55 (17.8)	49 (27.8)	6 (4.5)	<0.0001
Hypertension (*n*, (%))	215 (69.4)	118 (67.0)	97 (72.4)	0.312
Dyslipidemia (*n*, (%))	218 (70.3)	118 (67.0)	100 (74.6)	0.148
Duration of Diabetes (years)	10.5 ± 8.1	10.9 ± 8.3	9.9 ± 7.8	0.269
ARB or ACEi (*n*, (%))	153 (49.4)	79 (44.9)	74 (55.2)	0.071
Ca blocker (*n*, (%))	128 (41.4)	66 (37.5)	62 (46.3)	0.120
β blocker (*n*, (%))	22 (7.1)	9 (5.1)	13 (9.7)	0.119
MR antagonist (*n*, (%))	7 (2.3)	3 (1.7)	4 (3.0)	0.452
Statin (*n*, (%))	149 (48.1)	71 (40.3)	78 (58.2)	0.002
Ezetimibe (*n*, (%))	19 (6.1)	8 (4.5)	11 (8.2)	0.183
Other hypolipidemic agents (*n*, (%))	20 (6.5)	13 (7.4)	7 (5.2)	0.443
Anti-platelets (*n*, (%))	34 (11.0)	23 (13.1)	11 (8.2)	0.175
SU or Glinide (*n*, (%))	54 (17.5)	29 (16.5)	25 (18.7)	0.616
Metformin (*n*, (%))	146 (47.1)	83 (47.2)	63 (47.0)	0.980
DPP4i (*n*, (%))	200 (64.7)	115 (65.3)	85 (63.4)	0.246
SGLT2i (*n*, (%))	64 (20.6)	42 (23.9)	22 (16.4)	0.109
αGI (*n*, (%))	67 (21.6)	45 (25.6)	22 (16.4)	0.053
Pioglitazone (*n*, (%))	21 (6.8)	14 (8.0)	7 (5.2)	0.343
Insulin (*n*, (%))	64 (20.6)	37 (21.0)	27 (20.1)	0.851
GLP-1RA (*n*, (%))	28 (9.0)	15 (8.5)	13 (9.7)	0.720

The values are presented as mean ± SD, medians (range) or *n* (%). Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; ARB, angiotensin II receptor blocker; BMI, body mass index; Cr, creatinine; DPP4i, dipeptidyl peptidase 4 inhibitor; eGFR, estimated glomerular filtration rate; 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; PG, plasma glucose; SBP, systolic blood pressure; SGLT2i, sodium glucose cotransporter 2 inhibitor; SU, sulfonylurea; TG, triglyceride; UA, uric acid; uACR, urinary albumin to creatinine ratio; uL-FABPCR, urinary liver-type fatty acid-binding protein to creatinine ratio; αGI, alpha-glucosidase inhibitor.

activity and the uL-FABPCR (Table 2). Because it is well known that pharmacological interventions (including antihypertensive agents, statins, and hypoglycemic agents) influence albuminuria in patients with diabetes, we next performed multiple-regression analysis with the confirmed independent variables shown in Table 2 and the medications used. Conclusively, plasma HCII activity remained a significant preventive factor against the development of albuminuria, regardless of the medications taken (Table S2).

Association between the plasma HCII activity and the eGFR

We performed multiple-regression analysis for the determinants of eGFR in a cross-sectional study. Although we identified age, male sex, HbA1c, serum creatinine, and the duration of

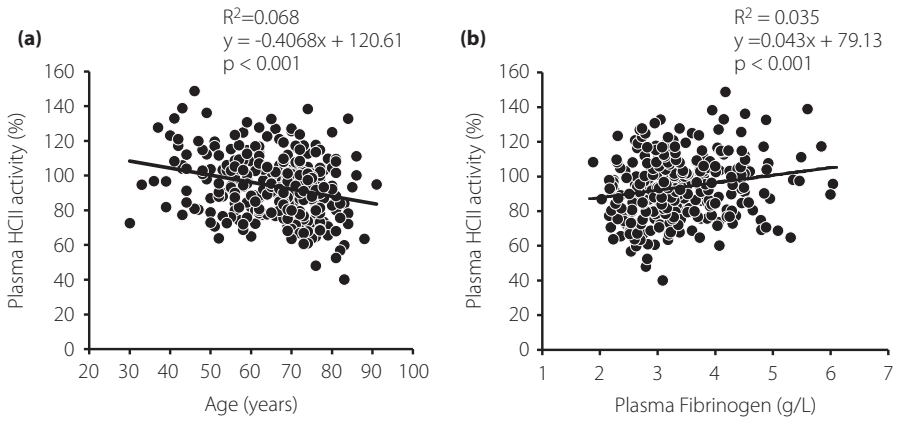

Figure 2 | Associations between Plasma HCII Activities and Age or Plasma Fibrinogen. (a) Scatterplots showing relationships between plasma the HCII activity and age. An inverse relationship was observed between the plasma HCII activity and age. (b) Scatterplots showing relationships between plasma HCII activities and plasma fibrinogen levels. A positive relationship was observed between plasma HCII activities and plasma fibrinogen levels. Data for all 176 males and 134 females enrolled in this study are shown.

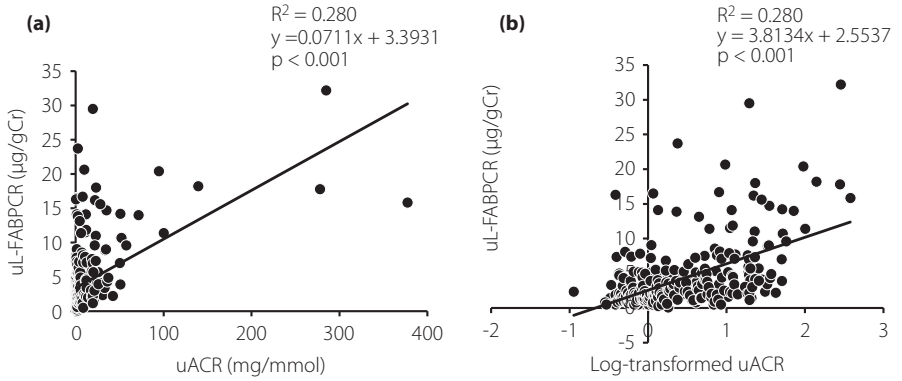

Figure 3 | Associations between Albuminuria and L-FABP. (a) Scatterplots between the uACR and uL-FABPCR values. (b) Scatterplots between the log-transformed uACR and uL-FABPCR values. Data for all 176 males and 134 females enrolled in this study are shown. Close and significantly positive relationships were observed between the urinary biomarkers examined.

diabetes as independent regulators of eGFR, we found no relationship between plasma HCII activities and eGFRs (Table S3).

Determinants of annual changes of the uACR and log-transformed uACR

For our longitudinal analysis, we evaluated 201 patients (115 males and 86 females; Table 3), among the 310 subjects in the cross-sectional study, for annual changes in their ⊿uACR and ⊿log-transformed uACR values. The plasma HCII activity was negatively correlated with the ⊿uACR ($R^2 = 0.058$, $P < 0.001$; Figure 5a) and ⊿log-transformed uACR ($R^2 = 0.024$, $P = 0.027$; Figure 5b). Next, we performed multiple-regression analysis to identify the independent determinants of ⊿uACR and ⊿log-transformed uACR. As shown in Table 4, the plasma HCII activity was an independent and negative contributor to increases in both the ⊿uACR and ⊿log-transformed uACR values ($P = 0.001$ and $P = 0.014$, respectively). Next, we statistically evaluated the contributions of plasma HCII activities and medications at baseline, as well as additional pharmacological agents (Table S4), during the longitudinal study. Table S5 shows that plasma HCII activity remained an independent protective factor against the annual development of albuminuria after adjustment for all medications.

In Table 4, the baseline SBP value was unexpectedly identified as a negative contributor to an increase in the ⊿log-transformed uACR ($P = 0.002$). We then investigated whether the annual change of SBP (⊿SBP) was associated with the ⊿log-transformed uACR. We found that ⊿SBP was significantly and positively associated with the ⊿log-transformed uACR (Figure S1). Therefore, we divided the 201 patients in

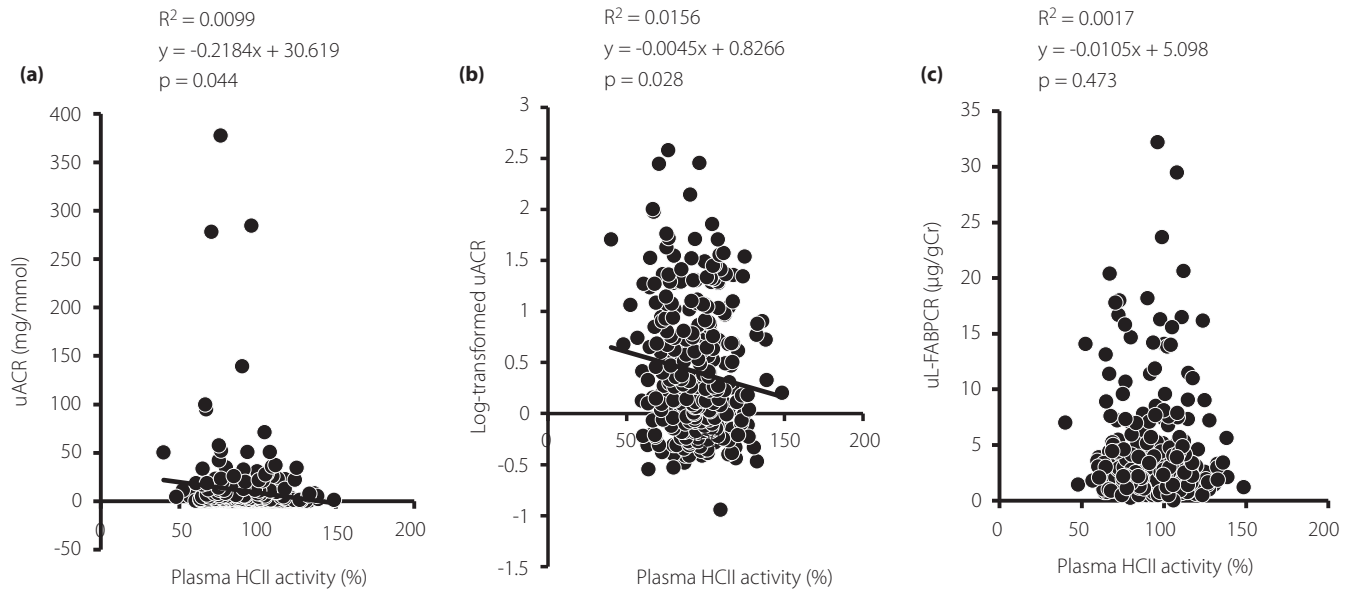

Figure 4 | Associations between Plasma HCII Activities and Urinary Biomarkers. (a) Scatterplots showing the relationships between plasma HCII activity and uACR values. (b) Scatterplots showing the relationships between plasma HCII activity and log-transformed uACR values. (c) Scatterplots showing the relationships between plasma HCII activity and uL-FABPCR values. Data for all 176 males and 134 females enrolled in this study are included in the scatter plots.

Table 2 | Multiple-regression analysis of determinants of urinary biomarkers in our cross-sectional study (176 males and 134 females)

Variables	uACR		Log-transformed uACR		uL-FABPCR	
	t Value	*P* value	*t* Value	*P* value	*t* Value	*P* value
Age	−0.36	0.716	0.93	0.356	1.56	0.121
Male	−2.59	0.010	−2.69	0.008	−2.10	0.036
BMI	0.19	0.853	0.92	0.360	2.19	0.030
SBP	2.95	0.003	4.66	<0.001	3.49	0.001
LDL-C	−0.37	0.708	−0.73	0.464	0.18	0.857
TG	−0.46	0.647	0.00	0.999	−0.48	0.634
HDL-C	−0.75	0.457	−0.83	0.410	−0.05	0.964
HbA1c	2.87	0.004	0.01	0.020	2.71	0.007
UA	0.30	0.761	−0.65	0.518	−0.46	0.642
Cr	3.41	0.001	3.37	0.001	3.39	0.001
Plasma fibrinogen	0.21	0.833	1.22	0.223	−0.04	0.965
Plasma HCII activity	−2.11	0.036	−2.76	0.006	−0.61	0.541
Current smoking	1.99	0.048	1.84	0.068	0.39	0.695
Hypertension	0.68	0.499	2.36	0.019	−0.70	0.484
Dyslipidemia	1.41	0.161	−0.42	0.672	−0.72	0.472
Duration of diabetes	1.89	0.060	1.52	0.129	1.77	0.077

the longitudinal analysis into two groups according to the baseline SBP (i.e., the SBP < 130 mm Hg group and the SBP ≥ 130 mm Hg group) and evaluated annual changes in each group. Although the SBP < 130 mm Hg group showed a slight SBP elevation at 1 year after baseline, the SBP ≥ 130 mm Hg group showed a significant annual reduction of SBP (Figure S2). During the longitudinal study, significantly more patients ($P = 0.048$) in the SBP ≥ 130 mm Hg group (11/118 patients) were treated with additional anti-hypertensive agents than in the BP < 130 mm Hg group (2/83 patients). Taken together, these results raise the possibility that BP management during this study influenced the ⊿log-transformed uACR.

Table 3 | Clinical characteristics of subjects at baseline in our longitudinal study

	Total	Male	Female	*P* value (male vs female)
Number of Subjects	201	115	86	
Type 1 diabetes mellitus	14	6	8	0.113
Age (years)	65.2 ± 10.9	64.6 ± 11.2	66.0 ± 10.4	0.353
BMI (m^2/kg)	25.7 ± 5.0	25.3 ± 4.7	26.2 ± 5.0	0.224
SBP (mmHg)	132.1 ± 16.2	132.3 ± 17.0	131.8 ± 15.1	0.813
LDL-C (mmol/L; mean [SD])	2.56 ± 0.74	2.48 ± 0.69	2.65 ± 0.79	0.118
TG (mmol/L; mean [SD])	1.49 ± 0.37	1.52 ± 0.77	1.45 ± 0.71	0.529
HDL-C (mmol/L; mean [SD])	1.43 ± 0.75	1.39 ± 0.40	1.49 ± 0.33	0.079
Casual PG (mmol/L; mean [SD])	8.01 ± 2.71	8.30 ± 2.82	7.62 ± 2.50	0.075
HbA1c (mmol/mol; mean [SD])	50.0 ± 8.22	49.9 ± 8.93	50.2 ± 7.2	0.817
HbA1c (%; mean [SD])	6.7 ± 0.8	6.7 ± 0.8	6.7 ± 0.7	0.817
UA (mmol/L; mean [SD])	0.31 ± 0.07	0.33 ± 0.07	0.28 ± 0.06	<0.0001
Cr (μmol/L; mean [SD])	57.3 ± 0.16.7	64.0 ± 16.2	48.3 ± 12.7	<0.0001
eGFR (ml/min/1.73 m^2)	76.5 ± 19.0	74.1 ± 20.4	77.0 ± 17.0	0.718
uACR (mg/mmol; median [IQR])	1.70 (0.84-5.50)	1.50 (0.81-5.70)	2.04 (0.97-5.41)	0.763
Log-transformed-uACR (mean [SD])	0.36 ± 0.55	0.35 ± 0.55	0.37 ± 0.55	0.745
uL-FABPCR (μg/gCr; median [IQR])	2.57 (1.6-4.46)	2.52 (1.59-4.45)	2.57 (1.6-4.55)	0.846
Plasma Fibrinogen (g/L; mean [SD])	3.47 ± 0.79	3.49 ± 0.81	3.44 ± 0.77	0.664
Plasma HCII Activity (%)	93.9 ± 18.0	93.3 ± 18.6	94.6 ± 17.2	0.625
Current Smoking (*n*, (%))	35 (17.4)	31 (30.0)	4 (4.7)	<0.0001
Hypertension (*n*, (%))	145 (72.1)	118 (67.0)	65 (75.6)	0.344
Dyslipidemia (*n*, (%))	139 (69.2)	76 (66.1)	63 (73.3)	0.274
Duration of Diabetes (years)	10.0 ± 7.6	10.6 ± 7.7	9.3 ± 7.4	0.200
ARB or ACEi (*n*, (%))	106 (52.7)	53 (46.1)	53 (61.6)	0.029
Ca blocker (*n*, (%))	81 (40.3)	40 (34.8)	41 (47.8)	0.065
β blocker (*n*, (%))	13 (6.5)	6 (5.2)	7 (8.1)	0.405
MR antagonist (*n*, (%))	5 (2.5)	3 (2.6)	2 (2.3)	0.899
Statin (*n*, (%))	101 (50.2)	52 (45.2)	49 (57.0)	0.099
Ezetimibe (*n*, (%))	11 (5.5)	4 (3.5)	7 (8.1)	0.151
Other hypolipidemic agents (*n*, (%))	14 (6.9)	10 (8.7)	4 (4.7)	0.265
Anti-platelets (*n*, (%))	24 (11.9)	16 (13.9)	8 (9.3)	0.319
SU or Glinide (*n*, (%))	33 (16.4)	20 (17.4)	13 (15.1)	0.667
Metformin (*n*, (%))	96 (47.8)	56 (48.7)	40 (46.5)	0.759
DPP4i (*n*, (%))	132 (65.7)	79 (68.7)	53 (61.6)	0.296
SGLT2i (*n*, (%))	32 (15.9)	22 (19.1)	10 (11.6)	0.150
αGI (*n*, (%))	47 (23.4)	35 (30.4)	12 (14.0)	0.006
Pioglitazone (*n*, (%))	18 (9.0)	12 (10.4)	6 (7.0)	0.396
Insulin (*n*, (%))	43 (21.4)	20 (17.4)	19 (22.1)	0.404
GLP-1RA (*n*, (%))	18 (9.0)	8 (7.0)	10 (11.6)	0.251

The values are presented as mean±SD, medians (range) or *n* (%). Abbreviations: ACEi, angiotensin-converting enzyme inhibitor; αGI, alpha-glucosidase inhibitor ARB, angiotensin II receptor blocker; BMI, body mass index; Cr, creatinine; DPP4i, dipeptidyl peptidase 4 inhibitor; eGFR, estimated glomerular filtration rate; 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; PG, plasma glucose; SBP, systolic blood pressure; SGLT2i, sodium glucose cotransporter 2 inhibitor; SU, sulfonylurea; TG, triglyceride; UA, uric acid; uACR, urinary albumin to creatinine ratio; uL-FABPCR, urinary liver-type fatty acid-binding protein to creatinine ratio.

Association between Plasma HCII activities and the medications used

Since most participants in this study were treated with various medications, it is possible that some of the medications used exerted considerable influences on plasma HCII activities. Thus, we performed multiple-regression analysis to determine which medications were associated with plasma HCII activities. Our analysis showed that insulin acted as the sole and negative pharmacological agent in terms of the plasma HCII activity (Table S6). Because we previously reported that low plasma HCII activity promotes insulin resistance and gluconeogenesis [21], low plasma HCII activity may exacerbate the severity of diabetes resulting in increase of insulin use.

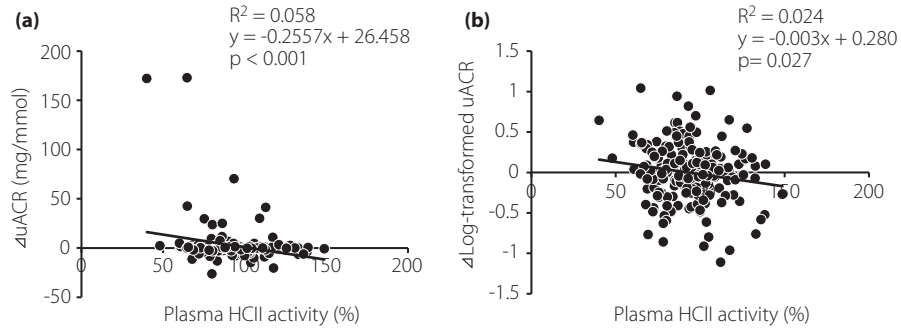

Figure 5 | Associations between Plasma HCII Activities and Annual Changes of Albuminuria. (a) Scatterplots showing the relationships between plasma HCII activity and ⊿uACR values. (b) Scatterplots showing the relationships between plasma HCII activity and log-transformed ⊿uACR values. Data for 115 males and 86 females enrolled in this study are included in the scatterplots.

Table 4 | Multiple-regression analysis of determinants of the annual changes in albuminuria found in our longitudinal study (115 males and 86 females)

Variables	⊿uACR		⊿Log-transformed uACR	
	t Value	*P* value	*t* Value	*P* value
Age	−0.45	0.675	−1.09	0.276
Male	−1.58	0.115	−1.32	0.188
BMI	−0.63	0.528	−0.67	0.507
SBP	−0.98	0.327	−3.17	0.002
LDL-C	−1.68	0.094	−0.30	0.764
TG	−0.28	0.782	0.10	0.923
HDL-C	−0.41	0.684	−0.01	0.992
HbA1c	−0.80	0.424	−0.02	0.981
UA	1.65	0.100	1.22	0.225
Cr	0.88	0.382	0.77	0.445
Plasma fibrinogen	1.69	0.093	2.12	0.035
Plasma HCII activity	−3.27	0.001	−2.47	0.014
Current smoking	0.18	0.858	1.57	0.119
Hypertension	0.64	0.521	0.11	0.915
Dyslipidemia	0.54	0.589	−0.12	0.905
Duration of diabetes	−0.23	0.822	−1.03	0.306

DISCUSSION

In this study, we found that plasma HCII activity was independently and inversely associated with albuminuria and its annual deterioration in patient with diabetes. The results obtained suggest the importance of HCII as a common regulatory factor for CVDs and DKD.

HCII prevents CVDs and insulin resistance via inactivation of the thrombin–PAR-1 system

The thrombin–PAR system promotes CVDs and insulin resistance, and HCII is an endogenous mammalian thrombin inhibitor; therefore, we hypothesized that HCII prevents CVDs and preserves insulin sensitivity. Indeed, we previously reported that low plasma HCII activity was associated with the development of atherosclerosis, cardiac remodeling, and hyperglycemia with insulin resistance in humans [13,14,16,18,21]. Moreover, we generated heterozygous HCII-deficient mice by a gene-targeting method and demonstrated that the HCII-deficient mice manifested exaggerated cardiovascular remodeling and enhanced insulin resistance with increased gluconeogenesis [15,19-21]. Taken together, the results indicate that HCII prevents CVDs and maintains glucose metabolism.

The thrombin–PARs system promotes DKD

Hypercoagulability is often found in diabetic patients [24], and Ay et al.[25] revealed that patients with type-2 diabetes mellitus and macroalbuminuria showed increased thrombin generation. The activation of PARs can promote the development of diabetic nephropathy by disrupting the glomerular-filtration barrier with endothelial and glomerular cell apoptosis and podocyte loss [8,26]. In addition, inactivation of PAR signaling can blunt lesion formation in patients with diabetic nephropathy [9-11]. These findings are consistent with the notion that the thrombin–PAR system plays a pivotal role in DKD development.

HCII was identified as a common negative regulating factor in the development of CVDs and CKD, including DKD

Albuminuria has been recognized as a pivotal biomarker of renal insufficiency and CVDs; thus, CKD and CVDs may share considerable common risk factors related to the development of organ damages. In addition to our previous results, the present data indicate that HCII might be one of the common, negative regulatory factors for the development of CVDs and CKD including DKD.

HCII specifically inactivated the thrombin–PARs system in the kidney according to dermatan sulfate localization

In this study, no correlation was found between plasma HCII activities and uL-FABPCR values. L-FABP is mainly

synthesized in tubular cells. L-FABP is regarded as a urinary tubular biomarker associated with structural and functional kidney damage [27]. uL-FABP levels predict adverse outcomes in patients with diabetic nephropathy [28,29], as well as acute kidney injury and CKD progression due to nondiabetic causes [30-32]. Panduru et al.[33] reported that increments of L-FABP seems to be connected with tubular injury rather than diabetes itself, as L-FABP levels correlated poorly with HbA1c levels. In addition, thrombin inhibition by HCII was activated via binding to dermatan sulfate proteoglycans, which are synthesized by glomerular epithelial cells and mesangial cells [34]. Thus, HCII may suppress thrombin activity in the glomerulus, but not in tubules, based on the localization of dermatan sulfate.

Plasma HCII activity and eGFR

In this study, no relationship was found between plasma the HCII activity and eGFR. Given that the eGFR is thought to be influenced by glomerular injury and various other factors (including tubular injury and circulatory insufficiency due to atherosclerosis), eGFR determination is multifactorial and complicated. Because our study demonstrated that plasma the HCII activity was specifically associated with glomerular injury represented by albuminuria, HCII may play a small part in eGFR regulation as one of many clinical factors.

Study limitations

A limitation of this study is that the current results cannot be extended to the general population because (i) we enrolled only patients with diabetes and (ii) we previously found that subjects without cardiovascular risk factors (including diabetes) had higher plasma HCII activity levels than subjects with one or more cardiovascular risk factors [14,21]. In addition, the renal-protective effects of HCII may differ between patients with type-1 diabetes mellitus or type-2 diabetes mellitus. Too few patients with type-1 diabetes mellitus were enrolled in this study to clarify this issue, and a large-scale investigation is required to assess and clarify the prognostic value of plasma HCII activity in the general population, as well as differences between patients with type-1 diabetes mellitus or type-2 diabetes mellitus.

The data generated in this study demonstrates that the plasma HCII activity served as a negative clinical factor for albuminuria development in patient with diabetes. Measuring the plasma HCII activity might enable the prediction and/or development of glomerular disease in patients with DKD at an early stage.

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DISCLOSURE

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

Table S1 | Multiple-Regression Analysis of Determinants of Normal-Ranged uACR in Our Cross-Sectional Study (111 Males and 82 Females)

Table S2 | Multiple-Regression Analysis Including Identified Variables and Medications as Determinants of Urinary Biomarkers in Our Cross-Sectional Study (176 Males and 134 Females)

Table S3 | Multiple-Regression Analysis of Determinants of eGFR in Our Cross-Sectional Study (176 Males and 134 Females)

Table S4 | List of Additional Medications Used During the Longitudinal Study

Table S5 | Multiple-Regression Analysis Including Identified Variables and Medications for Determinants of Annual Changes in Albuminuria of Our Longitudinal Study (115 Males and 86 Females)

Table S6 | Multiple-Regression Analysis (Including Medications) of Determinants of Plasma HCII Activities (176 Males and 134 Females)

Figure S1 | Scatterplots between ⊿SBP and ⊿log-transformed uACR in patients for whom data were used for longitudinal analysis. Data for 115 males and 86 females in this study are shown.

Figure S2 | Annual changes of SBP in patients for whom data were used for longitudinal analysis.