

Clinical Significance of Urinary Liver-Type Fatty Acid-Binding Protein in Diabetic Nephropathy of Type 2 Diabetic Patients

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concentrations in diabetic nephropathy of type 2 diabetes.

RESEARCH DESIGN AND METHODS

OBJECTIVE—Urinary liver-type fatty acid-binding protein (L-FABP) is a promising indicator of tubular but not glomerular damage. The aim of this study was to evaluate the clinical usefulness of urinary L-FABP as a prognostic biomarker in impaired diabetic nephropathy in type 2 diabetes.

RESEARCH DESIGN AND METHODS—This investigation involved a cross-sectional and longitudinal analysis of the relationship between urinary L-FABP levels and progressive nephropathy. Urinary L-FABP was measured with enzyme-linked immunosorbent assay. In the cross-sectional analysis, the association of urinary L-FABP, with the severity of diabetic nephropathy, was investigated in 140 patients with type 2 diabetes and in 412 healthy control subjects. Of the patients in the former study, 104 have been followed for 4 years. The progression of diabetic nephropathy was defined as progressive albuminuria, end-stage renal disease, or induction of hemodialysis.

RESULTS—Urinary L-FABP levels were progressively increased in subjects with normo-, micro-, or macroalbuminuria and further increased in patients with end-stage renal disease. In the longitudinal analysis, high urinary L-FABP levels were associated with the increase in albuminuria, progression to end-stage renal disease, or induction of hemodialysis. This was particularly demonstrated in the subgroup of patients without renal dysfunction ($n = 59$), where high urinary L-FABP levels were associated with the progression of diabetic nephropathy.

CONCLUSIONS—Urinary L-FABP accurately reflected the severity of diabetic nephropathy in type 2 diabetes, and its level was high in the patients with normoalbuminuria. Moreover, higher urinary L-FABP was a risk factor for progression of diabetic nephropathy.

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Liver-type fatty acid-binding protein (L-FABP) is expressed in the proximal tubules of the human kidney and participates in fatty acid metabolism (1–3). In one clinical study, urinary excretion of L-FABP was reported to offer potential as a clinical marker to screen for kidney dysfunction and thereby to

identify patients who are likely to experience deterioration of renal function in the future (4).

The current study evaluated the control reference values for urinary L-FABP in spot urine, and cross-sectional and longitudinal analyses were conducted on the clinical relevance of urinary L-FABP

Healthy subjects and patient selection

Reference values for urinary L-FABP in spot urine. To determine control reference values for urinary L-FABP in spot urine and to compare the levels of urinary L-FABP and urinary albumin of each diabetic nephropathy group with those of healthy control subjects, 70 volunteers from St. Marianna University School of Medicine Hospital (Kawasaki, Japan) and Senpo Tokyo Takanawa Hospital (Tokyo, Japan) and 342 subjects who underwent medical checkups at the health center of Dokkyo University School of Medicine (Tochigi, Japan) were examined to assess general physical health and clinical parameters of blood and urine.

Cross-sectional analysis. This study was carried out between March 2004 and September 2004, and 199 adult patients were recruited with type 2 diabetes from the outpatient clinics at the Department of Internal Medicine, St. Marianna University School of Medicine Hospital (Kawasaki, Japan). The inclusion criteria for the patients were as follows: no history of liver disease, primary kidney disease, cancer, or collagen disease and no hemodialysis. From the 199 patients, 140 were selected who fulfilled these criteria. Blood and spot urine samples were collected three times from all of the patients. Table 1 summarizes the clinical characteristics and laboratory findings of the patients.

Prospective observational follow-up study.

From the patients enrolled in cross-sectional analysis ($n = 140$), patients who were seen regularly at the outpatient clinic of St. Marianna University School of Medicine during 2004–2008 were recruited ($n = 104$). The patients underwent biochemical measurements such as urinary albumin and serum creatinine three times a year.

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Table 1—Clinical characteristics and laboratory findings of patients

	Albuminuria			End-stage renal failure	Between-group differences
	Normo	Micro	Macro		
N	64	30	27	19	
Sex (male/female)	42/22	18/12	13/14	15/4	NS
Age (years)	63 (29–84)	66 (44–84)	62 (41–82)	64 (47–77)	NS
Known diabetes duration (years)	11 (1–41)	15 (3–43)	15 (5–25)	14 (5–32)	NS
Diabetic retinopathy, n (%)	15 (23.4)	14 (46.7)	21 (77.8)	17 (89.5)	$\chi^2 = 38.2, P = 0.000$
SBP (mmHg)	133.7 ± 16.5	144.5 ± 16.5	140.3 ± 16.7	144.9 ± 15.1	$P = 0.002$
DBP (mmHg)	74.9 ± 11.9	74.1 ± 10.9	74.0 ± 9.6	69.8 ± 10.2	NS
Body weight (kg)	63.8 ± 14.4	63.6 ± 11.6	66.8 ± 14.5	62.9 ± 12.3	NS
BMI (kg/m ²)	24.4 ± 4.5	24.9 ± 3.9	25.7 ± 3.9	24.1 ± 3.0	NS
HbA _{1c} (%)	6.8 ± 0.9	7.4 ± 1.0	7.6 ± 1.3	6.3 ± 0.9	$P < 0.0001$
Glycemia (mmol/L)	7.90 ± 2.41	9.41 ± 3.53	8.81 ± 3.65	7.87 ± 2.32	NS
eGFR (mL/min/1.73 m ²)	75.9 ± 15.6	69.0 ± 18.8	49.4 ± 16.6	13.9 ± 5.5	$P < 0.0001$
Total cholesterol (mmol/L)	5.24 ± 0.67	5.07 ± 0.67	5.67 ± 1.06	4.73 ± 0.93	$P = 0.0024$
Urinary albumin (mg/g creatinine)*	13.1 (7.4–20.6)	51.4 (37.2–93.4)	920.3 (476.9–1,839.2)	1,860.5 (1,408.7–3,011.5)	$P < 0.0001$
Urinary L-FABP (μg/g creatinine)*	4.8 (2.5–8.1)	8.6 (5.0–12.5)	64 (22.8–120.7)	209.3 (160.7–407.3)	$P < 0.0001$
Concomitant medication					
Insulin, n (%)	22 (34.4)	14 (46.7)	12 (44.4)	13 (68.4)	NS
Lipid-lowering treatment, n (%)	26 (40.6)	11 (36.7)	14 (51.9)	8 (42.1)	NS
RAS blockade treatment, n (%)	20 (31.3)	14 (46.7)	20 (74.0)	18 (94.7)	$\chi^2 = 30.5, P = 0.000$

Data are means ± SD, median (range), or *median (IQR).

These studies were carried out according to the principles of the Declaration of Helsinki, and written informed consent was obtained from all of the patients. We obtained ethics approval for our study from the ethics committees.

Study procedure

Severity of diabetic nephropathy and urinary L-FABP. To evaluate progression of disease, patients were divided into four diabetic nephropathy stages based on the degree of albuminuria or renal function found in at least two of the three samples collected, as follows: normoalbuminuria (urinary albumin level <30 mg/g creatinine); microalbuminuria (urinary albumin level 30–300 mg/g creatinine); macroalbuminuria (urinary albumin level >300 mg/g creatinine); and end-stage renal failure (serum creatinine level >176.8 μmol/L). Urinary L-FABP levels in each group were compared with the L-FABP levels in all of the other groups (i.e., at each stage of diabetic nephropathy), as was the urinary level of albumin. Furthermore, the levels of those parameters of each diabetic nephropathy group were compared with those of 412 healthy control subjects.

Progression of diabetic nephropathy and urinary L-FABP. The primary end points were the development of microalbuminuria,

macroalbuminuria, end-stage renal failure, or induction of hemodialysis. The increase in albuminuria was evaluated by the degree of albuminuria found in at least two of the three samples collected and meant from normoalbuminuria to microalbuminuria or from microalbuminuria to macroalbuminuria. The patients were divided into two groups based on showing or not showing progress of diabetic nephropathy. The progression group was defined as the patients whose diabetic nephropathy was developed to the primary end points. Furthermore, the patients with estimated glomerular filtration rate (eGFR) >60 mL/min/1.73 m² at entry were selected from all patients followed for 4 years and were evaluated using the same analysis.

Measurements

ELISA for measurement of urinary L-FABP. Urinary levels of L-FABP in spot urine samples were measured by ELISA using the Human L-FABP ELISA Kit (CMIC, Tokyo, Japan) (4). The detection limit was 3.0 μg/L. As for inter- and intra-assay coefficient of variations (CVs), eight replicate measurements were made on each of three different urine samples with L-FABP concentrations of 27.0, 74.0, and 261 μg/L, respectively. Intra-assay variabilities were 4.8, 3.1, and

2.6%, respectively. To determine interassay variabilities, each of the three urine samples was measured on eight successive days, and results were 4.4, 3.5, and 2.6%, respectively.

Clinical parameters of blood and urine.

Serum creatinine and total cholesterol, plasma glycemia, and glycosylated hemoglobin (HbA_{1c}) were measured in the blood samples. In the spot urine samples, urinary creatinine and albumin were measured.

The levels of urinary parameters in spot urine samples were expressed as a ratio to the level of urinary creatinine. GFR was estimated using the new equation proposed by the Japanese Society of Nephrology as follows: eGFR (mL/min/1.73 m²) = 194 × Cr^{-1.094} × Age^{-0.287} × 0.739 (if female) (5).

The three values of each parameter were measured in the samples on three different days. For each individual, the median of the three values was used for statistical analysis.

Statistical analysis in both studies

Normally distributed variables were expressed as means ± SD or median (range). The levels of urinary parameters were given as the median (interquartile range [IQR]). To compare two groups, the unpaired *t* test (parametric distributions)

or the Mann-Whitney *U* test (nonparametric distributions) was used for the unpaired data. Differences in the levels of urinary parameters between each diabetic nephropathy group and the control group were analyzed by the Steel method. The levels of urinary parameters in the four diabetic nephropathy groups (i.e., at the different stages of diabetic nephropathy) were compared using the Steel-Dwass method after the Kruskal-Wallis test had been performed. In the four groups, normally distributed variables were compared in a one-way ANOVA and categorical variables were compared using the χ^2 test. To determine control reference values of urinary L-FABP, the urinary L-FABP levels were analyzed using the logarithmic-transformed data. These statistical analyses were performed using SAS 8.2 software (SAS Institute, Cary, NC). *P* values <0.05 were considered to be statistically significant.

Receiver operating characteristic (ROC) for clinical parameters were plotted to predict the progression of diabetic nephropathy. Cox regression analysis was performed to determine the predictor for the progression of diabetic nephropathy 4 years later. The presence of albuminuria including microalbuminuria, systolic blood pressure (SBP), diastolic blood pressure (DBP), HbA_{1c}, age, sex, and the use of renin-angiotensin system (RAS) inhibitor, which are known as risk factors in progression of diabetic nephropathy, and a higher level of urinary L-FABP (than upper limit of reference value) were selected as variables. The

odds ratios and 95% confidence intervals were calculated. These statistical analyses were performed using Stat Flex 5.0 software (Artec, Osaka, Japan). *P* values <0.05 were considered to be statistically significant.

RESULTS

Reference values for urinary L-FABP in spot urine

In the 412 healthy volunteers, the mean value of urinary L-FABP in spot urine, determined from the logarithmic-transformed data (log L-FABP), was 1.6 $\mu\text{g/g}$ creatinine, with individual values ranging from 0.3 $\mu\text{g/g}$ creatinine (mean $- 2$ SD) to 8.4 $\mu\text{g/g}$ creatinine (mean $+ 2$ SD). The log L-FABP *P* values showed a lognormal distribution across the 412 control subjects (data not shown).

Severity of diabetic nephropathy and urinary parameters

Urinary levels of L-FABP (Fig. 1A) and albumin (Fig. 1B) in the patients with normoalbuminuria were significantly higher than those in normal control subjects (*P* < 0.05). The levels of urinary L-FABP and urinary albumin in each diabetic nephropathy group were significantly different from the levels in all of the other groups and significantly increased according to the severity of diabetic nephropathy (*P* < 0.05).

Prospective observational follow-up study

Clinical characteristics in each group are shown in Table 2. In all of the patients

followed for 4 years (*n* = 104), there were significant differences in known diabetes duration, DBP, eGFR, urinary L-FABP, and urinary albumin between the two groups (Table 2). A parameter with the primary large area under the ROC curve (AUC) for predicting the progression of diabetic nephropathy was urinary albumin (0.857), and the secondary large AUC was urinary L-FABP (0.849; Table 3). The difference between the AUCs for the two parameters was not significant (*P* = 0.876). In Cox regression analysis, a higher level of urinary L-FABP (than upper limit of reference value of urinary L-FABP, 8.4 $\mu\text{g/g}$ creatinine) at the start of the study was associated with the progression of diabetic nephropathy and DBP and HbA_{1c} at the start were inversely associated with it (Table 4). Urinary albumin was associated at the start of the study with the progression of diabetic nephropathy. However, after adjustment for known progression promoters and high values of urinary L-FABP, there was no association between urinary albumin and progression of diabetic nephropathy.

In the patients with eGFR >60 mL/min/1.73 m², there were significant differences in urinary L-FABP and urinary albumin between the two groups (Table 2). A parameter with the primary large AUC for predicting the progression of diabetic nephropathy was urinary L-FABP (0.761), whereas the secondary large AUC was urinary albumin (0.675; Table 3). The difference between the AUCs for two analyses was not significant (*P* = 0.451). In Cox regression analysis, a

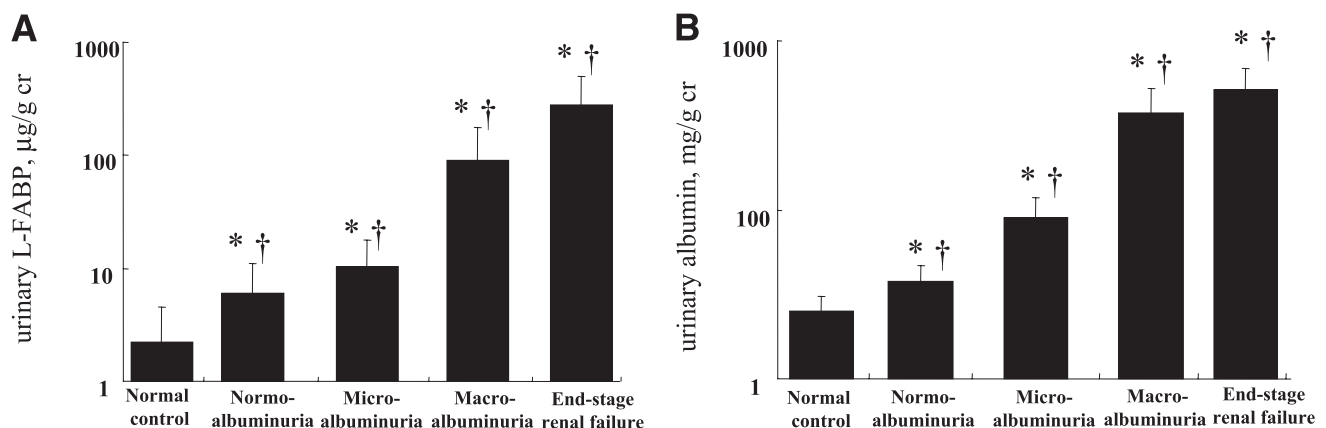


Figure 1—A: Relationship between urinary L-FABP levels and progression of diabetic nephropathy. The level of urinary L-FABP increased significantly according to the severity of diabetic nephropathy. Urinary L-FABP in the patients with normoalbuminuria was significantly higher than in normal control subjects. **P* < 0.05, compared with normal control group; †*P* < 0.05, compared with all the other groups of diabetic nephropathy. cr, creatinine. B: Relationship between urinary albumin levels and progression of diabetic nephropathy. The level of urinary albumin increased according to the severity of diabetic nephropathy. Urinary albumin in the patients with normoalbuminuria was significantly higher than in normal control subjects. **P* < 0.05, compared with normal control group; †*P* < 0.05, compared with all the other groups of diabetic nephropathy.

Table 2—Clinical parameters for subgroups of patients in the prospective follow-up study, according to the presence of progression of diabetic nephropathy

Parameter	Group		P value
	Progression	Nonprogression	
All patients followed for 4 years			
N	47	57	
Sex (male/female)	31/16	33/24	NS
Age (years)	64 ± 11	63 ± 10	NS
Known diabetes duration (years)	16.0 ± 8.3	12.4 ± 8.0	P = 0.035
SBP (mmHg)	138.1 ± 16.6	139.1 ± 17.0	NS
DBP (mmHg)	71.1 ± 10.1	75.3 ± 8.5	P = 0.016
HbA _{1c} (%)	7.0 ± 1.2	7.1 ± 1.0	NS
Glycemia (mmol/L)	8.27 ± 2.67	8.39 ± 1.11	NS
eGFR (mL/min/1.73 m ²)	40.9 ± 26.1	71.6 ± 22.0	P = 0.000
Total cholesterol (mmol/L)	5.16 ± 0.92	5.10 ± 0.65	NS
Urinary albumin (mg/g creatinine)*	1,150.6 (353.0–2,301.7)	22.6 (8.8–50.6)	P = 0.000
Urinary L-FABP (μg/g creatinine)*	77.9 (16.9–181.6)	6 (3.3–12.1)	P = 0.000
Severity of diabetic nephropathy, n			
Normoalbuminuria	10	32	
Microalbuminuria	1	19	
Macroalbuminuria	19	4	
Serum creatinine more than 176.8 mmol/L, n	17	2	
RAS blockade treatment, n (%)	34 (72%)	31 (54%)	NS
Patients with eGFR >60 mL/min/1.73 m ²			
N	14	45	
Sex (male/female)	10/4	27/18	NS
Age (years)	61 ± 16	62 ± 10	NS
Known diabetes duration (years)	16.1 ± 8.9	12.8 ± 8.4	NS
SBP (mmHg)	130.1 ± 16.0	137.9 ± 18.3	NS
DBP (mmHg)	74.6 ± 12.1	75.8 ± 8.9	NS
HbA _{1c} (%)	7.8 ± 1.0	7.2 ± 0.9	NS
Glycemia (mmol/L)	8.40 ± 2.87	8.50 ± 2.35	NS
eGFR (mL/min/1.73 m ²)	73.7 ± 8.6	79.3 ± 16.5	NS
Total cholesterol (mmol/L)	5.21 ± 0.66	5.19 ± 0.60	NS
Urinary albumin (mg/g creatinine)*	27.5 (19.0–379.9)	15.4 (7.6–44.3)	P = 0.018
Urinary L-FABP (μg/g creatinine)*	12.1 (10.1–19.9)	6.0 (3.3–9.6)	P = 0.003
Severity of diabetic nephropathy, n			
Normoalbuminuria	9	29	
Microalbuminuria	1	14	
Macroalbuminuria	4	2	
RAS blockade treatment, n (%)	5 (36%)	22 (49%)	NS

Data are means ± SD, n (%), or *median (IQR).

higher level of urinary L-FABP (than upper limit of reference value of urinary L-FABP, 8.4 μg/g creatinine) at the start of the study was associated with progression of diabetic nephropathy (Table 4).

CONCLUSIONS—The results of this study indicate that the level of urinary L-FABP accurately reflected the severity of diabetic nephropathy and was significantly higher in the patients with type 2 diabetes who had normoalbuminuria than in normal control subjects. In the prospective study, urinary L-FABP higher

than the upper limit of reference value was a risk factor for progression of diabetic nephropathy. Therefore, urinary L-FABP appears to be a useful marker for the detection of early-stage diabetic nephropathy and for the prediction of the progression of diabetic nephropathy.

Chronic hypoxia is recognized to be an aggravating factor that is common to many kidney diseases (6). In the early phase of diabetic nephropathy without glomerular dysfunction, chronic hyperglycemia causes oxidative stress and sympathetic denervation of the kidney

because of autonomic neuropathy (7), which provokes microvasculature damage and leads to tubulointerstitial hypoxia. Therefore, chronic hypoxia appears to play a dominant pathogenic role both in triggering early-stage diabetic nephropathy and in promoting progression of diabetic nephropathy. Recently, it was reported that tubular hypoxia upregulated the expression of the L-FABP gene in the kidney and increased the urinary excretion of L-FABP from the proximal tubules (8). Thus, in early-stage diabetic nephropathy, it is possible that chronic hypoxia could have induced an increase in urinary

Table 3—AUC for predicting the progression of diabetic nephropathy in parameters

	AUC	
	All patients followed for 4 years	Patients with eGFR >60 mL/min/1.73 m ²
Age (years)	0.51	0.53
Known diabetes duration (years)	0.641	0.636
SBP (mmHg)	0.511	0.615
DBP (mmHg)	0.644	0.56
HbA _{1c} (%)	0.522	0.565
Glycemia (mmol/L)	0.542	0.579
eGFR (mL/min/1.73 m ²)	0.797	0.549
Total cholesterol (mmol/L)	0.491	0.456
Urinary albumin (mg/g creatinine)	0.857	0.675
Urinary L-FABP (μg/g creatinine)	0.849	0.761

excretion of L-FABP, even in the absence of albuminuria. There may be a tubulointerstitial abnormality in those patients with higher urinary L-FABP levels.

Urinary albumin reflects glomerular damage, and urinary L-FABP reflects tubulointerstitial damage. In diabetic nephropathy, both glomerular damage and tubulointerstitial damage progress to end-stage renal failure. In this prospective follow-up study, in addition to urinary albumin, urinary L-FABP was also considered to have potential as a clinical marker for identifying the patients who are likely to experience deterioration of renal function. Although urinary L-FABP

level was significantly correlated with urinary albumin level in all of the patients, urinary L-FABP level did not correlate with the urinary albumin level in the subgroup of patients who had eGFR >60 mL/min/1.73 m² (data not shown). Therefore, we submit that urinary L-FABP can reflect the pathophysiological condition of diabetic nephropathy that is not possible with urinary albumin and that the combination of urinary albumin and urinary L-FABP could be a good marker, not only for early diagnosis of diabetic nephropathy but also for risk stratification and assessment of the severity of diabetic nephropathy. In the recent clinical prospective observational

study of the patients with type 1 diabetes, urinary L-FABP was also reported to be independent predictors of microalbuminuria and death (9).

In the current study, there was a finding that conflicted with presumably confirmed reports. Although urinary albumin levels in the progression group were significantly higher than in the non-progression group and urinary albumin levels predicted the progression of diabetic nephropathy in unadjusted analysis, urinary albumin concentration was not associated with a higher risk of progression of diabetic nephropathy after adjustment for conventional risk factors and high values of urinary L-FABP. As for the cause, first, it was considered that the progression of diabetic nephropathy was defined not only as a progression to the end-stage renal failure or induction of hemodialysis but also as the increase in albuminuria in this study. Diabetic nephropathy advanced to a next higher stage in patients with lower urinary albumin levels or normoalbuminuria as well as in those with massive urinary albumin levels.

In summary, the current study found that the level of urinary L-FABP accurately reflected the severity of diabetic nephropathy. In addition to urinary albumin, the measurement of L-FABP in urine provides a suitable biomarker for the early detection and monitoring of progression of diabetic

Table 4—Cox regression analysis using the progression of diabetic nephropathy unadjusted and after adjustment for high value of urinary L-FABP at entry, presence of albuminuria at entry, SBP, DBP, HbA_{1c}, age, sex, and RAS blockade treatment

	Unadjusted (univariate)			Adjusted (multivariate)		
	Hazard ratio	95% CI	P value	Hazard ratio	95% CI	P value
All patients followed for 4 years						
High value of urinary L-FABP at entry	5.206	2.425–21.883	0.001	7.285	2.425–21.883	0.000
Presence of albuminuria at entry	2.073	1.002–4.288	0.049	0.736	0.300–1.809	NS
SBP	0.975	0.805–1.181	NS	0.924	0.745–1.147	NS
DBP	0.632	0.444–0.898	0.011	0.593	0.402–0.876	0.009
HbA _{1c}	0.624	0.440–0.887	0.008	0.666	0.466–0.952	0.026
Age	1.275	0.910–1.787	NS	1.173	0.821–1.675	NS
Sex	1.625	0.802–3.291	NS	1.245	0.557–2.785	NS
RAS blockade treatment	1.402	0.703–2.796	NS	1.566	0.683–3.590	NS
Patients with eGFR >60 mL/min/1.73 m ²						
High value of urinary L-FABP at entry	5.014	1.399–17.978	0.013	9.458	2.241–39.916	0.002
Presence of albuminuria at entry	0.942	0.316–2.810	NS	0.404	0.091–1.807	NS
SBP	0.809	0.592–1.106	NS	0.758	0.450–1.276	NS
DBP	0.837	0.479–1.463	NS	0.854	0.371–1.965	NS
HbA _{1c}	1.213	0.711–2.071	NS	1.129	0.625–2.038	NS
Age	0.902	0.570–1.427	NS	0.865	0.477–1.569	NS
Sex	0.620	0.195–1.979	NS	0.509	0.123–2.099	NS
RAS blockade treatment	0.654	0.219–1.951	NS	1.048	0.275–3.997	NS

nephropathy in clinical practice. To gather definitive support that urinary L-FABP is an appropriate biomarker in predicting progression of diabetic nephropathy at various stages of nephropathy, further research in a large-sized multicenter trial is needed in which adequate numbers of patients in each subset will be available for study.

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