Predictive Effects of Urinary Liver-Type Fatty Acid-Binding Protein for Deteriorating Renal Function and Incidence of Cardiovascular Disease in Type 2 Diabetic Patients Without Advanced Nephropathy.

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OBJECTIVE—To improve prognosis, it is important to predict the incidence of renal failure and cardiovascular disease in type 2 diabetic patients before the progression to advanced nephropathy. We investigated the predictive effects of urinary liver-type fatty acid–binding protein (L-FABP), which is associated with renal tubulointerstitial damage, in renal and cardiovascular prognosis.

RESEARCH DESIGN AND METHODS—Japanese type 2 diabetic patients (n = 618) with serum creatinine ≤1.0 mg/dl and without overt proteinuria were enrolled between 1996 and 2000, and followed up until 2011. Baseline urinary L-FABP was measured with an enzyme-linked immunosorbent assay. The primary end points were renal and cardiovascular composites (hemodialysis, myocardial infarction, angina pectoris, stroke, cerebral hemorrhage, and peripheral vascular disease). The secondary renal outcomes were the incidence of a 50% decline in estimated glomerular filtration rate (eGFR), progression to an eGFR <30 mL/min/1.73 m², and the annual decline rate in eGFR.

RESULTS—During a 12-year median follow-up, 103 primary end points occurred. The incidence rate of the primary end point increased in a stepwise manner with increases in urinary L-FABP. In Cox proportional hazards analysis, the adjusted hazard ratio in patients with the highest tertile of urinary L-FABP was 1.93 (95% CI 1.13–3.29). This relationship was observed even when analyzed separately in normoalbuminuria and microalbuminuria. Patients with the highest tertile of urinary L-FABP also demonstrated a higher incidence of the secondary renal outcomes.

CONCLUSIONS—Our results indicate that urinary L-FABP may be a predictive marker for renal and cardiovascular prognosis in type 2 diabetic patients without advanced nephropathy.

Patients with type 2 diabetes are at a high risk for the progression to end-stage renal disease (ESRD) and incidence of cardiovascular disease (CVD), both of which are life-threatening complications (1). To improve prognosis in diabetic patients, it is clinically important to identify patients at high risk for these disorders as early as possible and initiate disease management in a timely and appropriate manner.

ESRD and CVD share a number of clinical features and risk factors that are important therapeutic targets. Microalbuminuria is well known to be a common risk factor of ESRD and CVD, and a reduction of urinary albumin excretion (UAE) via any intervention results in a reduced future incidence of these disorders (2,3). However, many patients still develop ESRD and CVD despite improvements in their outcome resulting from recent aggressive multifactorial management (4–6). Thus, we need to explore new predictive markers for these disorders that are independent of UAE.

Renal dysfunction, also referred to as chronic kidney disease (CKD), is also an important predictive factor for ESRD and CVD that is independent of increases in UAE (7,8). There is a growing body of evidence suggesting that tubulointerstitial damage, as well as glomerular damage, contributes to a decline in renal function (9). Thus, measuring factors that relate to the risk of renal tubulointerstitial damage may be potentially useful for identifying patients at higher risk for ESRD and CVD.

Liver-type fatty acid–binding protein (L-FABP), an intracellular carrier protein of free fatty acids, is expressed in the liver and kidney. In the kidney, the expression of L-FABP is predominantly located in the proximal tubules. The high levels of urinary L-FABP were previously suggested to be associated with renal tubulointerstitial damage because excessive reabsorption of free fatty acids into the proximal tubules induces tubulointerstitial damage (10–12). Based on these findings, we conducted a long-term observational study to investigate whether urinary levels of L-FABP were predictive for the progression of renal dysfunction and cardiovascular disease in type 2 diabetic patients without advanced nephropathy.
Predictive effects of urinary L-FABP

of renal dysfunction and incidence of CVD in patients with type 2 diabetes without advanced nephropathy.

RESEARCH DESIGN AND METHODS

Subject recruitment
Japanese patients with type 2 diabetes were recruited from participants that were registered in the Shiga Prospective Observational Follow-up Study between 1996 and 2000 (13). Patients with cancer, recent occurrences of CVD within the past year, infectious disease, collagen disease, and non-diabetic kidney disease, as confirmed by a renal biopsy, were excluded from the study. After obtaining written informed consent, each individual provided a 24-h urine sample and fasting blood sample at baseline. The serum and urine samples were kept at –80°C, if they were not analyzed immediately. In this study, patients with normoalbuminuria/microalbuminuria and serum creatinine (Cr) ≤1.0 mg/dL were eligible. Based on the UAE rate (UAER) at baseline, patients were classified as having normoalbuminuria (UAER <20 μg/min), microalbuminuria (20 ≤ UAER <200 μg/min), or overt proteinuria (UAER ≥200 μg/min). Serum concentrations of Cr were measured via an enzymatic method. Finally, 618 patients with normoalbuminuria (n = 422) and microalbuminuria (n = 196) were enrolled and followed up until the end of 2011 or the first occurrence of any renal and cardiovascular composite endpoint. The participants annually underwent standardized clinical examinations and biochemical tests during the follow-up period. HbA1c levels were presented as National Glycohemoglobin Standardization Program values, according to the recommendations of the Japanese Diabetes Society (14). The study protocol and informed consent procedure were approved by the Ethics Committee of Shiga University of Medical Science.

Measurement of urinary L-FABP
Urinary concentrations of L-FABP were measured using a two-step sandwich enzyme-linked immunosorbent assay (15), and all stored samples obtained at baseline were simultaneously measured in 2002. In this study, the baseline levels of urinary L-FABP in each individual were obtained from one urine sample, as described above. The sensitivity of this assay was >3.0 μg/L. Both of the intra- and interassay coefficients of variation were <10%, respectively. Urinary concentrations of Cr were also measured via an enzymatic method. Urinary excretion levels of L-FABP were expressed as micrograms per gram of Cr.

Follow-up evaluation
The primary end point was the first occurrence of any of the renal and cardiovascular composites, which were as follows: initiation of chronic hemodialysis and the occurrence of myocardial infarction, angina pectoris, stroke, cerebral hemorrhage, peripheral vascular disease (PAD), and death from cardiovascular causes. Myocardial infarction was defined as a clinical presentation characterized by typical symptoms, electrocardiographic changes associated with an elevation of cardiac biomarkers, and angiographic evidence of coronary thrombosis. Angina pectoris was defined as the presence of responsible lesions detected by imaging studies with a history of typical chest pain or electrocardiographic changes and invasive cardiovascular interventions. Stroke, including ischemic stroke and cerebral hemorrhage, was defined as a persistent focal neurologic symptom in which the onset was sudden and was not due to trauma or a tumor and where the responsible lesion was detected by imaging studies. PAD was defined as revascularization with typical symptoms such as cold feet or intermittent claudication. At the annual physical examination of this cohort, we directly examined patients and checked their medical records to identify the onset of primary end points. In a fatal case, the medical record was reviewed by physicians to identify the cause of death. If the cause of death was unclear, it was not counted as a death from cardiovascular cause.

In evaluating the secondary outcomes, we separately assessed CVD events and renal secondary outcomes. In regards to secondary renal outcomes, we assessed two categorical outcomes: a 50% decline in the estimated glomerular filtration rate (eGFR) from baseline and the progression to stage 4 CKD (eGFR <30 mL/min/1.73 m²) and one outcome as a continuous variable, the annual rate of decline in eGFR over the study period. eGFR was calculated using the simplified prediction equation proposed by the Japanese Society of Nephrology (16): eGFR (mL/min/1.73 m²) = 194 × [age (years)]⁻⁰.₂⁰⁸ × [serum Cr (mg/dL)]⁻¹⁰⁹⁴ × 0.7⁹³ (for female). At baseline, all participants had an eGFR >60 mL/min/1.73 m². In the analysis of the annual rate of decline in eGFR, only patients that were observed over 3 years were used in the estimation of the rate of decline in eGFR. The annual rate of decline in eGFR over the course of the study was determined from the slope of each individual from the linear regression analysis and expressed in mL/min/1.73 m²/year.

Statistical analysis
Data are expressed as mean ± SD or median (IQR), where appropriate. Patients were divided into tertiles according to the urinary levels of L-FABP at baseline. Statistical significance of the differences among the three subgroups was determined via a x² test for categorical variables, and an ANOVA followed by the Tukey-Kramer test for normally distributed variables or the Kruskal-Wallis test for nonnormally distributed continuous variables. The incidence rate per 1,000 person-years for each outcome was calculated. The cumulative incidence was estimated by using the Kaplan-Meier method and compared with the log-rank test. The follow-up time was censored if any primary end point occurred or if the patient was unavailable for follow-up. The adjusted hazard ratio (HR) for each outcome was evaluated by using a Cox proportional hazards regression model. In this analysis, the known cardiovascular risk factors were age, sex, BMI, HbA1c, total cholesterol, triglycerides, HDL cholesterol, hypertension, use of renin-angiotensin system (RAS) inhibitors, systolic and diastolic blood pressure, past history of CVD, stage of nephropathy (or log UAER for log urinary L-FABP), and eGFR at baseline. The difference of the annual decline rate in eGFR after controlling for the effect of systolic BP and log AER was assessed with the ANCOVA model. All analyses were performed with the SPSS software package (version 11; SPSS Inc., Chicago, IL). A two-sided P value <0.05 was considered statistically significant.

RESULTS—The baseline characteristics of the 618 patients and three subgroups stratified by urinary levels of L-FABP at baseline are presented in Table 1. Age, duration of diabetes, HbA1c, total cholesterol, systolic BP, hypertension, use of RAS inhibitors, urinary AER, microalbuminuria, urinary β₂-microglobulin, and past history of CVD were significantly
Urinary L-FABP (–point)

Incidence rates of the primary end point were significantly different among the three subgroups (P < 0.001, log-rank test). As shown in Fig. 1, the cumulative incidences of the primary end point were significantly different among the three subgroups (P < 0.0001, log-rank test). The risk for the primary end point was evaluated by using the Cox proportional hazards model (Table 2). When adjusted for known cardiovascular risk factors, the HR in the highest tertile of urinary L-FABP was 1.93 (95% CI 1.13–3.29). Using log urinary L-FABP as a continuous variable, instead of the tertiles of urinary L-FABP, the HR of log urinary L-FABP for primary end points was 2.16 (95% CI 1.23–3.79) after adjusting for age, sex, log UAER, and eGFR at baseline, and 1.79 (1.06–3.01) after adjusting for known cardiovascular risk factors.

Effects of urinary L-FABP on secondary renal outcomes

The incidence rates per 1,000 person-years for a 50% decline in eGFR from baseline in the three subgroups were 4.8 in the lowest tertile, 6.0 in the middle tertile, and 18.3 in the highest tertile. Also, the incidence rates for the progression to stage 4 CKD (eGFR <30 ml/min/1.73 m²) were 1.8 in the lowest tertile, 2.4 in the middle tertile, and 11.1 in the highest tertile. The adjusted HRs for these secondary renal outcomes were significantly higher in the highest tertile (Table 2). The annual rate of decline in eGFR (ml/min/1.73 m²/year) was −1.31 (95% CI −0.46 to −2.33) in the lowest tertile, −1.65 (−1.02 to −2.25) in the middle tertile, and −1.80 (−1.05 to −3.21) in the highest tertile (P = 0.002, Kruskal-Wallis test), and there was a significant effect of urinary L-FABP on the annual decline rate in eGFR after controlling for the effect of systolic BP and log AER (F = 3.54, P = 0.03, ANCOVA). In addition, patients in the highest tertile of urinary L-FABP showed the highest incidence of a 50% decline in eGFR, which was associated with the highest incidence of CVD. The cumulative incidence of CVD was significantly higher in patients with a 50% decrease in eGFR than those without it (P = 0.034, log-rank test).

Risk of urinary L-FABP according to the stage of diabetic nephropathy

We finally investigated the incidence rates and HRs for the primary end point in the subgroups stratified according to the levels of urinary L-FABP and the stages of diabetic nephropathy at baseline. As shown in Table 3, the incidence rates and HRs adjusted from known cardiovascular risk factors increased with increasing stages of nephropathy and urinary L-FABP levels. Interestingly, the adjusted HR of the subgroups, categorized according to the highest tertile of urinary L-FABP, was significantly higher even in patients with normoalbuminuria.

Table 1—Baseline clinical characteristics of all patients with type 2 diabetes and the three subgroups stratified according to the levels of urinary L-FABP

<table>
<thead>
<tr>
<th>Variable</th>
<th>All</th>
<th>≤5.0</th>
<th>5.0–9.5</th>
<th>&gt;9.5</th>
<th>P value&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>n</td>
<td>618</td>
<td>206</td>
<td>206</td>
<td>206</td>
<td>NS</td>
</tr>
<tr>
<td>Male (%)</td>
<td>54.9</td>
<td>49.5</td>
<td>58.3</td>
<td>56.8</td>
<td>NS</td>
</tr>
<tr>
<td>Age (years)</td>
<td>59±10</td>
<td>58±10</td>
<td>58±10</td>
<td>62±10</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>BMI (kg/m²)</td>
<td>23.4±3</td>
<td>23.5±3</td>
<td>23.4±3</td>
<td>23.4±3</td>
<td>NS</td>
</tr>
<tr>
<td>Duration (years)</td>
<td>11±8</td>
<td>10±7</td>
<td>10±8</td>
<td>13±9</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diet/OHA/insulin (%)</td>
<td>25/52/23</td>
<td>31/52/17</td>
<td>26/56/18</td>
<td>17/50/33</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Total cholesterol (mg/dL)</td>
<td>213±36</td>
<td>220±34</td>
<td>209±34</td>
<td>212±38</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>HDL cholesterol (mg/dL)</td>
<td>56±46–66</td>
<td>57±47–67</td>
<td>54±46–67</td>
<td>55±47–64</td>
<td>NS</td>
</tr>
<tr>
<td>Triglycerides (mg/dL)</td>
<td>98±71–143</td>
<td>98±71–148</td>
<td>96±69–141</td>
<td>98±63–143</td>
<td>NS</td>
</tr>
<tr>
<td>Systolic BP (mmHg)</td>
<td>129±14</td>
<td>127±14</td>
<td>132±14</td>
<td>134±13</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Diastolic BP (mmHg)</td>
<td>76±10</td>
<td>76±9</td>
<td>77±9</td>
<td>76±11</td>
<td>NS</td>
</tr>
<tr>
<td>Hypertension (%)</td>
<td>46.9</td>
<td>40.7</td>
<td>45.1</td>
<td>54.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Using RAS inhibitors (%)</td>
<td>14.2</td>
<td>11.1</td>
<td>10.2</td>
<td>19.9</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Past history of CVD (%)</td>
<td>10.0</td>
<td>8.2</td>
<td>7.3</td>
<td>14.6</td>
<td>&lt;0.05</td>
</tr>
<tr>
<td>Urinary AER (µg/min)</td>
<td>11±7–27</td>
<td>8±5–15</td>
<td>12±7–28</td>
<td>16±9–43</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Microalbuminuria (%)</td>
<td>31.7</td>
<td>18.9</td>
<td>32.5</td>
<td>43.7</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>eGFR (ml/min/1.73 m²)</td>
<td>88±18</td>
<td>87±18</td>
<td>89±17</td>
<td>87±19</td>
<td>NS</td>
</tr>
<tr>
<td>Urinary β2-microglobulin (µg/g Cr)</td>
<td>120±81–206</td>
<td>93±69–136</td>
<td>122±82–183</td>
<td>175±106–369</td>
<td>&lt;0.01</td>
</tr>
<tr>
<td>Urinary L-FABP (µg/g Cr)</td>
<td>7.2±4.2–11.5</td>
<td>3.4±2.3–4.3</td>
<td>7.2±6.0–8.4</td>
<td>14±11.4–20.6</td>
<td>&lt;0.01</td>
</tr>
</tbody>
</table>

Data are expressed as mean ± SD for normally distributed continuous variables or median (IQR) for skewed continuous variables. AER, albumin excretion rate; OHA, oral hypoglycemic agent. *Differences between the three subgroups were compared with a χ² test for categorical variables and ANOVA for continuous variables.
Secondary end points

- Lowest tertile group (Cr).
- Differences between groups were com-

Three groups strati

ic incidence of primary end points of the

Solid line, highest tertile group (fi

CONCLUSIONS—The present long-

term observational study on type 2

diabetic patients without advanced ne-

phropathy revealed that higher urinary

levels of L-FABP were associated with
deteriorating renal function and a higher

incidence rate of CVD. These associations

were observed in those with normoalbumi-

nuria as well as those with micro-

albuminuria, when separately analyzed

according to the stages of diabetic nep-

phropathy. Thus, these findings sug-

gest that urinary L-FABP can be used as

a biomarker for predicting future renal

dysfunction and incidence of CVD in

type 2 diabetic patients with an early

stage of nephropathy, in addition to

albuminuria.

Renal dysfunction is reported to cor-

relate with the degree of tubulointerstitial
damage (9). Although albuminuria per se

reflects glomerular damage and subse-

quently induces renal tubulointerstitial
damage, other factors and mechanisms,

independent of albuminuria, must be in-

volved in the development of tubulointer-

stitial damage under diabetic conditions.

In fact, a recent study reported on cases

where renal function rapidly declined

without an increase in UAE (17). Urinary

levels of L-FABP have been reported to be

associated with the histological severity of

renal tubulointerstitial lesions in human

(15) and animal studies (18,19). Our

studies investigating the association be-

tween urinary L-FABP levels and the pro-

gression of diabetic nephropathy revealed

that urinary L-FABP levels were associ-

ated with the histological severity of

renal tubulointerstitial lesions. Therefore,

we postulate that the progression of

renal dysfunction is due to the pro-

gression of tubulointerstitial damage.

Furthermore, these results sug-

gest the importance of tubulointerstitial
damage in the development of renal dys-

function under diabetic conditions.

In the current study, we focused on

the predictive effects of urinary L-FABP

deteriorating renal function and the

onset of CVD in type 2 diabetic patients

with early stages of nephropathy. Pre-

viously, there have been several clinical

studies investigating the association be-

tween urinary L-FABP levels and the pro-

gression of diabetic nephropathy that

mainly focused on the progression of

nephropathy based on UAE. In a 4-year

prospective cohort study on 54 patients

with type 2 diabetes, Kamijo-Ikemori

et al. (20) reported that higher urinary

L-FABP levels were associated with the

progression of glomerular damage and

the onset of CVD. In contrast, Nielsen et al.

(21) reported that higher urinary L-FABP

levels predicted all-cause mortality in 165

patients with type 1 diabetes and nor-

moalbuminuria, independent of UAE and

other established risk factors. Our

findings strengthen these previous results

and provide further evidence that urinary

L-FABP is a predictive biomarker for renal

dysfunction and the onset of CVD in di-

abetic patients.

However, Nielsen et al. (22) recently

reported that urinary L-FABP levels are

not related to a rapid decline in GFR.
in a 3-year intervention study on 63 type 1 diabetic patients with overt proteinuria. Massive albuminuria per se induces tubulo-interstitial damage and then leads to renal dysfunction. Therefore, the effects of urinary L-FABP on tubulo-interstitial lesions and decline in GFR may disappear with an increase in albuminuria, such as overt proteinuria. Further investigation is needed to clarify this argument.

CKD, even a mild decline in renal function, is well acknowledged as an important risk factor for cardiovascular morbidity and mortality. A number of diabetic patients with renal dysfunction experience an onset of CVD before they initiate chronic hemodialysis. Also, our study demonstrated a higher incidence of CVD in patients who showed a 50% decline in eGFR during the follow-up than those who did not show a 50% decline.

There are some limitations in this study that must be addressed. In general practice, we do not perform renal biopsies in diabetic patients unless the complication of other renal diseases is suspected. Thus, we could not investigate the correlation between the urinary L-FABP levels and renal lesions in this study. Our study was designed as an observational follow-up study, and not an intervention trial. The treatment protocol for patients in this cohort was not controlled, and the influence of potential confounders during the observation period was not analyzed. Furthermore, the time-dependent changes of urinary L-FABP levels during the follow-up period were not assessed. Urinary L-FABP may be modified by any intervention (23,24). Thus, a further study is required to answer the important question of whether the changes of urinary L-FABP levels are associated with the prognosis in diabetic patients.

In conclusion, the current study indicated that the high levels of L-FABP in urinary excretion were associated with deteriorating renal function and the high incidence of CVD in patients with type 2 diabetes. This association was markedly observed even in patients with normoalbuminuria. Thus, measurements of urinary L-FABP, in addition to albuminuria, may be clinically useful for the early identification of diabetic patients without advanced nephropathy and at a higher risk for renal disease and CVD. In addition, these results suggest the importance of tubulo-interstitial damage in the development of renal dysfunction and CVD under diabetic conditions.

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S.A. designed the study protocol, researched data, and wrote the manuscript. M.H. designed the study protocol, contributed to discussion, and reviewed and edited the manuscript. D.K. searched data, contributed to discussion, and reviewed and edited the manuscript. T.S., K.I., and S.K. researched data. A.K., T.U., and H.M. contributed to discussion and reviewed and edited the manuscript. S.A. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

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References

Table 3—Incidence rates and adjusted HRs for primary end points in patient subgroups stratified according to the levels of urinary L-FABP and stages of diabetic nephropathy

<table>
<thead>
<tr>
<th>Urinary L-FABP</th>
<th>Lowest tertile</th>
<th>Middle tertile</th>
<th>Highest tertile</th>
</tr>
</thead>
<tbody>
<tr>
<td>Incidence rate</td>
<td>Lowest tertile</td>
<td>Middle tertile</td>
<td>Highest tertile</td>
</tr>
<tr>
<td>(1,000 person-years)</td>
<td>7.8</td>
<td>10.9</td>
<td>21.7</td>
</tr>
<tr>
<td>Normal albuminuria</td>
<td>17.8</td>
<td>25.7</td>
<td>31.0</td>
</tr>
<tr>
<td>Adjusted HR (95% CI)*</td>
<td>Normal albuminuria</td>
<td>1 (reference)</td>
<td>1.49 (0.72–3.09)</td>
</tr>
<tr>
<td>Microalbuminuria</td>
<td>1.72 (0.68–4.38)</td>
<td>2.70 (1.26–5.81)</td>
<td>2.18 (1.08–4.40)</td>
</tr>
</tbody>
</table>

*The HRs were adjusted for age, sex, BMI, Hba1c, total cholesterol, log triglycerides, log HDL cholesterol, hypertension, use of RAS inhibitors, systolic and diastolic blood pressure, past history of CVD, and eGFR in the Cox proportional hazards model.