Urinary Liver-Type Fatty Acid-Binding Protein Predicts Progression to Nephropathy in Type 1 Diabetic Patients

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OBJECTIVE — Urinary liver-type fatty acid-binding protein (u-LFABP) is a marker of tubulointerstitial inflammation and has been shown to be increased in patients with type 1 diabetes and is further increased in patients who progress to micro- and macroalbuminuria. Our aim was to evaluate u-LFABP as a predictor of progression to micro- and macroalbuminuria in type 1 diabetes.

RESEARCH DESIGN AND METHODS — From an inception cohort of 277 patients, u-LFABP, adjusted for urinary creatinine (enzyme-linked immunosorbent assay), was measured in 24-h urine samples from 165 normoalbuminuric patients 9.6 ± 3.5 (mean ±SD) years after onset of type 1 diabetes. The outcome measured was development of persistent micro- or macroalbuminuria or death.

RESULTS — Patients were followed for a median of 18 (range 1–19) years; 39 progressed to microalbuminuria, 8 of those progressed further to macroalbuminuria, and 24 died. In a Cox regression model, baseline log u-LFABP levels predicted the development of microalbuminuria, adjusted for known risk factors (sex, age, A1C, systolic and diastolic blood pressure, albumin excretion rate, serum creatinine, and smoking) (hazard ratio [HR] 2.3 [95% CI 1.1–4.6]) and log u-LFABP predicted mortality (adjusted HR 3.0 [1.3–7.0]). u-LFABP (above versus below the median) predicted the development of macroalbuminuria (adjusted HR 2.6 [1.2–5.4]). As a continuous variable, u-LFABP tended to predict macroalbuminuria (HR 1.9, $P = 0.2$), but numbers were small.

CONCLUSIONS — High levels of the tubular inflammation marker u-LFABP predict the initiation and progression to diabetic nephropathy and all-cause mortality, independent of urinary albumin excretion rate and other established risk factors.

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During the past few decades the prevention and treatment of late complications in diabetes have improved dramatically. The focus on prevention of late diabetes complications has changed and now involves tight glycemic control, reducing blood pressure, and lipid lowering (1), but, despite these efforts, diabetic patients still develop complications.

Approximately 30–40% of all patients with diabetes develop diabetic nephropathy (1), and this is the leading cause of end-stage renal disease in the Western world. In addition, diabetic nephropathy is associated with a higher risk of other complications (cardiovascular disease, neuropathy, and retinopathy) and with an increase in all-cause mortality (2).

It is known that tubulointerstitial damage plays an important role in diabetic nephropathy (3). Therefore, it would potentially be beneficial if albuminuria, as a marker of glomerular damage, could be supplemented by a marker of tubular damage to provide a more complete status of the kidney injury. This could help us first to more accurately predict the patients at risk of developing diabetic nephropathy and second to provide a better and possibly different treatment for diabetic nephropathy.

Liver-type fatty acid-binding protein (LFABP) is an intracellular carrier protein that is expressed in the proximal tubules in the human kidney and the liver (4). It has been demonstrated to be a marker of tubular damage (5).

Previously we have, in a cross-sectional setting, shown that urinary (u)-LFABP is increased in diabetic patients, even before they develop signs of glomerular damage, microalbuminuria or macroalbuminuria (6). This indicates that tubular damage is present at an early stage of diabetic kidney damage, even before the development of microalbuminuria. u-LFABP has, to our knowledge, not yet been studied in a prospective cohort study in type 1 diabetic patients.

To extend our previous cross-sectional findings we have evaluated the prognostic value of u-LFABP in a prospective study of type 1 diabetic patients who were still in a normoalbuminuric state. We have thereby been able to investigate whether u-LFABP contributes further to the established predictors for development of microalbuminuria or macroalbuminuria and the risk of death.

RESEARCH DESIGN AND METHODS

— We recruited an inception cohort of 277 patients from the outpatient clinic at Steno Diabetes Center from 1979 to 1984 for a prospective study of risk factors for development of complications. Figure 1 shows the design of the study and a flow chart. The patients had newly diagnosed type 1 diabetes. They were treated according to guidelines described earlier (7) and were followed yearly with blood and urine samples (8).

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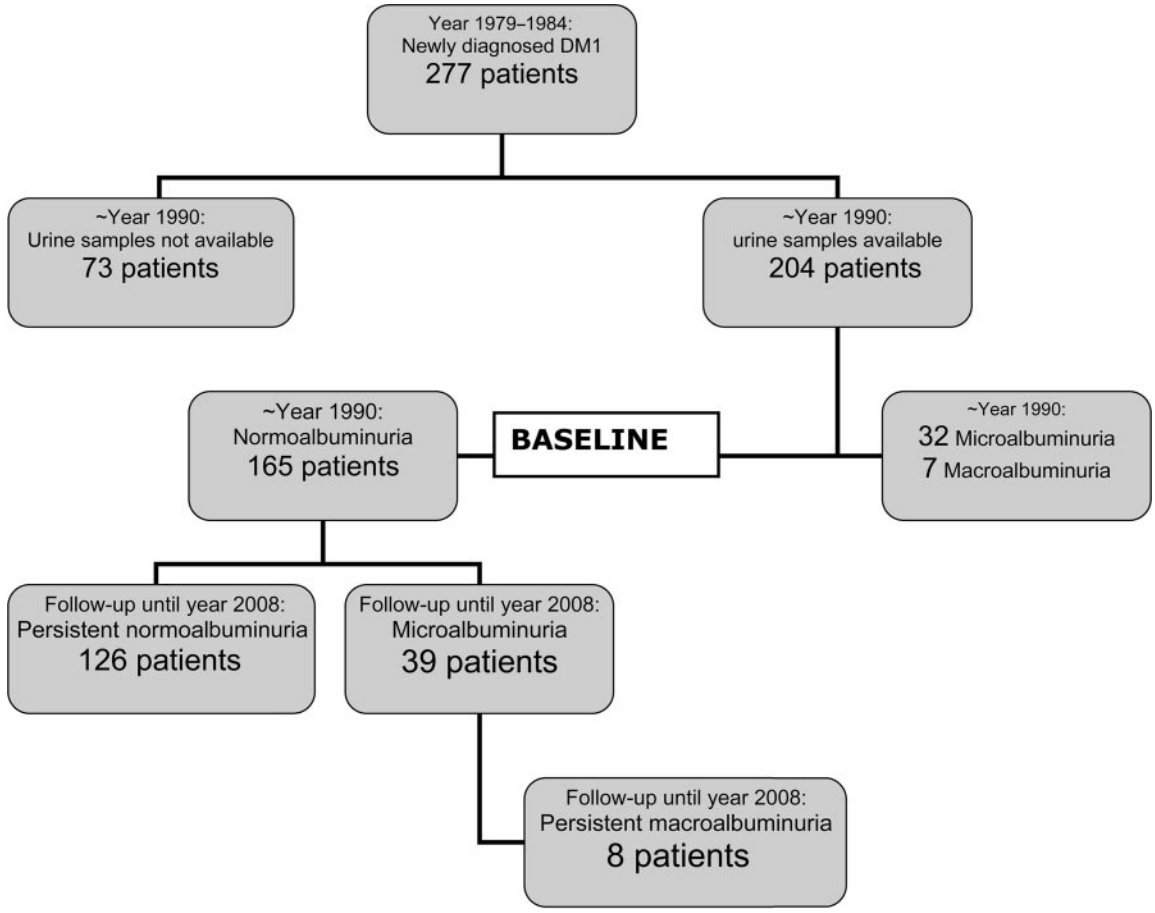

Figure 1—*Design of the study.*

Unfortunately, urine samples from before 1990 were lost, so in our analyses we included urine samples from 1990 and onward. Urine samples were available for 204 patients and of these 165 patients were normoalbuminuric. In 2008 we decided to analyze these urine samples for u-LFABP. u-LFABP was measured in the first urine sample available after 1990, 9.6 ± 3.5 years (mean ±SD) after onset of type 1 diabetes, and this was considered baseline in the present study (Fig. 1). Hereafter, the patients were followed regarding end points for a median (range) of 18 (1–19) years. The primary outcomes in our study were time to development of microalbuminuria or macroalbuminuria or death and were evaluated from the time of the patients' first urine sample after 1990 and until 2008 or the last available urine sample in patients lost to follow-up. Vital status was assessed in 2008 in the National Registry for all patients . All clinical baseline data were calculated as a mean of observations from the baseline year for each patient.

As far as possible, urinary albumin excretion per 24 h was measured yearly in each patient. Persistent microalbuminuria and persistent macroalbuminuria were defined as a urinary albumin excretion rate (UAER) between 30 and 300 and >300 mg/24 h, respectively, in at least two of three consecutive samples. All urine samples were collected as 24-h samples.

Arterial blood pressure was measured at least once per year with a standard mercury sphygmomanometer and was performed with the patient in a seated position after ~10 min of rest. Smoking history was determined via questionnaire, and patients were classified as smokers if they were smoking >1 cigarette per day.

Urine samples were stored at −20°C until u-LFABP analysis in 2009. u-LFABP was measured in a two-step sandwich enzyme-linked immunosorbent assay (9) and adjusted for u-creatinine. The inter- and intra-assay variations was 6.8 and 8.2%, respectively.

All patients provided informed consent for the participation in the study.

Statistical analysis

Data are means ± SD for the normally distributed variables. Variables with skewed distribution are given as geometric means (95% CI). Cumulative incidences of microalbuminuria and macroalbuminuria were calculated using Cox regression analyses. In Table 1 differences between groups are analyzed using ANOVA.

A Cox model for each of the three end points (development of microalbuminuria, development of macroalbuminuria, or death) was analyzed in all 165 patients. Microalbuminuria was most frequent but was a surrogate end point, and thus we analyzed the development of macroalbuminuria separately. If a patient was categorized as "microalbuminuric" or "macroalbuminuric" (two of three samples), he or she would not be recategorized if they later regressed in albuminuria. Patients were followed to 2008 or were censored at time of death. Patients who were lost to follow-up were counted in the analyses using the results of their last available urine sample. By using the Na-

Table 1—*Baseline characteristics of the 165 normoalbuminuric type 1 diabetic patients who had their u-LFABP measured in 1990 or later, divided into groups according to their later development of microalbuminuria or macroalbuminuria or persistent normoalbuminuria*

	Persistent normoalbuminuria	Only microalbuminuria	Macroalbuminuria	Progressors	*P* value*
n (male/female)	62/64	22/9	6/2	28/11	0.05
Age (years)	37 ± 12	41.7 ± 2.4	43 ± 9	41 ± 15	0.18
Diabetes duration (years)	8.6 ± 3.4	8.4 ± 1.8	12.1 ± 4.5	9.0 ± 2.8	0.05
Systolic blood pressure (mmHg)	122 ± 15	128 ± 16	138 ± 20	130 ± 17	0.02
Diastolic blood pressure (mmHg)	77 ± 8	79 ± 9	86 ± 14	81 ± 10	0.01
UAER (mg/24 h)	8 (7–9)	11 (8–14)	12 (7–20)	11 (9–14)	0.02
A1C (%)	8.2 ± 1.1	8.6 ± 1.7	9.1 ± 0.9	8.7 ± 1.6	0.02
Serum creatinine (μmol/l)	72 ± 11	69 ± 12	70 ± 13	69 ± 12	0.48
u-LFABP/creatinine [(pg/ml)/(mg/dl)]	9.6 (7.8–11.8)	13.4 (8.4–21.3)	12.6 (8.4–21)	13.2 (8.8–19.9)	0.35

Data are means ± SD or geometric means (95% CI). The microalbuminuria group does not include patients who later developed macroalbuminuria. Progressors include those who progressed to microalbuminuria and macroalbuminuria. *Overall difference between normoalbuminuric, microalbuminuric, and macroalbuminuric groups compared by ANOVA.

tional Register, vital status was available for all patients by the end of 2008.

A Cox regression model was used to analyze u-LFABP as an explanatory variable for the development of microalbuminuria and macroalbuminuria or death. Subsequently the model was adjusted for known risk factors: sex, age, A1C, systolic and diastolic blood pressure, UAER, serum creatinine, and smoking. Schoenfeld residuals were plotted against time to test for violation of the proportional hazards assumption, and linearity of the log hazard function was assessed by plotting the Martingale residuals against the covariates. We looked for interaction between u-LFABP and sex, A1C, or urinary albumin-to-creatinine ratio but found no evidence of interaction. Although the number of variables can be discussed, we found the model to work well for microalbuminuria with the listed and usually applied covariates. Models with fewer variables gave very similar hazard ratios (HRs) for predicting microalbuminuria; subsequently we applied the same model to the other end points.

u-LFABP is reported as a categorical variable in Fig. 2 for the presentation of a Kaplan-Meier plot. Receiver operating char-

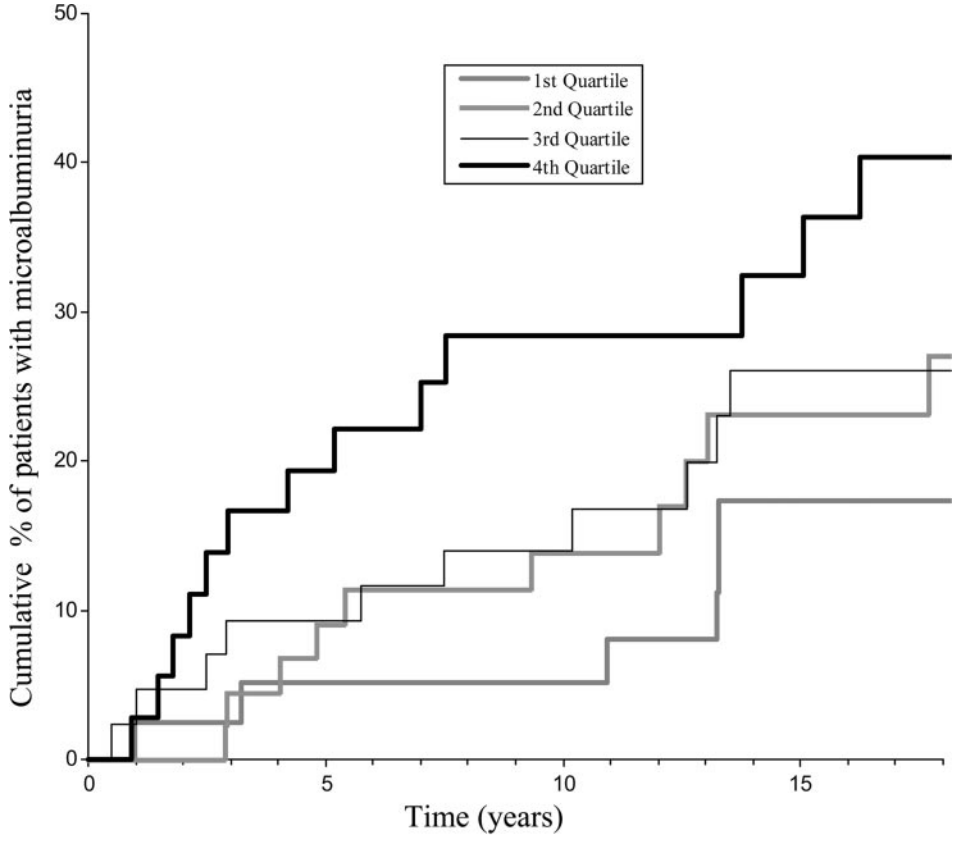

Figure 2—*Kaplan-Meier plot: elevated baseline u-LFABP levels in 165 normoalbuminuric type 1 diabetic patients predict progression to microalbuminuria. Quartiles with limits: u-LFABP/creatinine: 4.7, 10.9 and 21.3 (pg/ml)/(mg/dl). P = 0.02 for overall difference.*

acteristic curves were calculated in SPSS 15 (SPSS, Chicago, IL), assuming nonparametric distribution of parameters for SE of area.

Statistical significance was assumed for $P < 0.05$. Data were analyzed using SPSS 15.0.

RESULTS — At baseline, 165 patients of the 204 patients followed since 1979–1984 were persistently normoalbuminuric, and our later analyses and reported data are based on these patients (see Fig. 1 for flow chart). Mean ± SD age at baseline was 38 ± 12.6 years. Diabetes duration at baseline was 9.6 ± 3.5 years. The patients were followed for a median (range) of 18 (1–19) years. Successful follow-up until the end point or year 2008 was available in 90.3% of all patients. During follow-up to 2008, 39 patients had developed persistent microalbuminuria, and of these 8 patients progressed further to persistent macroalbuminuria. During follow-up, 24 patients died. The cumulative incidence of microalbuminuria was mean 27% (95% CI 20–35), of macroalbuminuria was 6% (2–10), and of death was 17% (11–23).

Baseline characteristics of the patients, when divided into groups according to the later development of microalbuminuria and macroalbuminuria or persistent normoalbuminuria, can be seen in Table 1. Patients who later developed microalbuminuria or macroalbuminuria had higher systolic and diastolic blood pressure than the patients with persistent normoalbuminuria ($P < 0.05$). A1C was progressively greater in the macroalbuminuric and microalbuminuric groups than in the normoalbuminuric group ($P = 0.02$). UAER was also increased in the patients later developing microalbuminuria or macroalbuminuria; however, there were no significant difference between the groups. As demonstrated in Table 1, u-LFABP was increased in the microalbuminuric and macroalbuminuric patients compared with the patients with persistent normoalbuminuria; however, this result was not statistically significant ($P = 0.17$ and $P = 0.52$).

In a Cox regression model (Table 2), baseline log u-LFABP levels predicted microalbuminuria when adjusted for known risk factors (age, sex, A1C, systolic and diastolic blood pressure, UAER, serum creatinine, and smoking) with a HR of 2.3 (95% CI 1.1–4.6). When u-LFABP was analyzed as a predictor of microalbuminuria using receiver operating curve analysis, we found that with addition of u-LFABP to the known risk factors (age, sex, A1C, systolic and diastolic blood pressure, UAER, serum creatinine, and smoking), the area under the curve increased only slightly from 0.80 (0.71–0.89) to 0.81 (0.72–0.91).

Table 2—*Cox regression: u-LFABP predicts the development of microalbuminuria when adjusted for known risk factors*

	Odds ratio (95% CI)	*P* value
Sex (male)	4.19 (1.62–10.87)	0.003
Age (year)	1.02 (0.99–1.05)	0.274
A1C (%)	2.00 (1.36–2.95)	<0.001
Systolic blood pressure (mmHg)	1.03 (0.995–1.06)	0.100
Diastolic blood pressure (mmHg)	0.99 (0.94–1.04)	0.758
Log (urinary albumin [mg]/24 h)	12.36 (2.58–59.32)	0.002
Log (u-LFABP [ng/ml]/urinary creatinine)	2.28 (1.14–4.58)	0.021

The analyses includes sex, age, A1C, systolic and diastolic blood pressure, UAER, u-LFABP, serum creatinine (not shown), and smoking (not shown).

As demonstrated in a Kaplan-Meier plot in Fig. 2, u-LFABP divided into quartiles predicts the development of microalbuminuria ($P = 0.024$). The HR from the first to fourth quartile is 5.8 ($P = 0.004$).

As a continuous variable, u-LFABP tended to predict the development of macroalbuminuria (HR 1.9), but this result was not statistically significant ($P = 0.2$), most likely because numbers were small ($n = 8$). When analyzed as a categorical variable u-LFABP [above versus below the median = 10.9 (pg/ml)/(mg/dl creatinine)] predicted the development of macroalbuminuria (HR 2.6 [95% CI 1.2–5.4]; adjusted for risk factors, see above).

During follow-up, 24 patients died. As a continuous variable, high levels of u-LFABP were associated with a significant increased risk of mortality (adjusted HR 3.0 [95% CI 1.3–7.0].

CONCLUSIONS — In our study u-LFABP predicted the future development of microalbuminuria and death in normoalbuminuric type 1 diabetic patients from an inception cohort study. The patients were followed for a median of 18 years, and the cumulative incidence of microalbuminuria and macroalbuminuria was mean 27% (95% CI 20–35) and 6% (2–10), respectively.

Previously, we demonstrated that u-LFABP is increased already in normoalbuminuric type 1 diabetic patients, indicating a "tubular phase" in the pathogenesis to diabetic kidney damage (6). However, this was done in a cross-sectional study, which in its design has some limitations, and thus nothing can be concluded on the time perspective between elevated u-LFABP and progression to microalbuminuria and macroalbuminuria. With the design of the present study, we have been able to demonstrate that high levels of u-LFABP are evident even before the development of microalbuminuria and hence at an early state where the glomerular damage is not detectable (albumin excretion rate not elevated), but where the tubules are affected (elevated u-LFABP). In Table 2 it is seen that elevated urinary albumin excretion in the normal range is also a strong predictor of progression to microalbuminuria. However, our aim was to supplement urinary albumin as a measure rather than replace it. If patients are followed over time, it is possible that changes in the markers within the normal range could add predictive value; however, we aimed to improve prediction of prognosis from a single point in time.

In an abstract presented at the American Society of Nephrology meeting in 2009 Kamijo et al. (10) reported that u-LFABP predicts a decrease in estimated glomerular filtration rate in type 2 diabetic patients with diabetic nephropathy. This finding supports our results and indicates that u-LFABP is a promising marker in not only type 1 but also type 2 diabetic patients. In a study of nondiabetic patients with chronic kidney disease, similar results were found: kidney function in patients with u-LFABP levels above the median deteriorated faster than that in patients with u-LFABP below the median during 1 year of follow-up (11). Tamm-Horsfall protein, another marker of tubular damage, is produced in the thick ascending limb of Henle. Tamm-Horsfall protein has been found to be predictive of cardiovascular death and uremia in type 1 but not type 2 diabetic patients (12). This finding again supports

the hypothesis that tubular markers could be added to albuminuria in the risk assessment of the development of diabetic nephropathy.

In the present study, we found that u-LFABP predicted death. It is not possible to define the causality, but it is most likely that the effect of u-LFABP is mediated through the development of elevated urinary albumin excretion. We found that patients who died had a significantly higher incidence of microalbuminuria or macroalbuminuria than patients who survived during follow-up (41 vs. 21%, $P = 0.027$).

By studying renal biopsy specimens with immunohistochemical staining, it has been shown that u-LFABP excretion is closely associated with structural and functional tubular kidney damage (13). This finding was confirmed in patients with chronic kidney disease including minimal change nephrotic syndrome, nephrosclerosis, lupus nephritis, and diabetic nephropathy (14). In a recent experimental study in transgenic mice, it has been shown that u-LFABP accurately reflects the degree of tubulointerstitial damage and is dynamic as a measure; it increases and decreases, reflecting damage and repair of the tubular cells (15). In accordance with these results, we previously reported that u-LFABP excretion is reduced when type 1 diabetic patients with diabetic nephropathy are treated with renoprotective treatment such as the ACE inhibitor lisinopril (6). This finding leads to speculations on u-LFABP as a monitor of renoprotective treatment; however, more studies are needed to confirm this.

Our results show that high levels of u-LFABP predict the development of microalbuminuria and diabetic nephropathy. However, this does not determine the mechanism or the role of LFABP: it has been hypothesized that LFABP is a protective protein (16), but previous studies have, to our knowledge, not been able to confirm this hypothesis (17). u-LFABP as a continuous variable did not predict macroalbuminuria, but it did as a categorical variable. Because of the low number of events ($n = 8$), the analysis has to be interpreted with caution, but it is in line with the prediction of microalbuminuria.

The present study has some limitations. The patients only had their u-LFABP measured in one 24-h urine sample, and the urine samples were stored at −20°C for ~18 years before analyses. Another limitation is the loss of urine samples before 1990 (Fig. 1). However, this most likely results in a underestimation of the predictive power of u-LFABP as patients most susceptible to renal disease, probably having the most tubular damage, already had developed microalbuminuria or macroalbuminuria and were excluded. The strengths of our study are that all patients were followed for vital status and >90% were followed to 2008 or the development of a renal end point.

In summary, we demonstrate that u-LFABP is elevated at an early stage, even before any clinical signs of glomerular damage are detectable, confirming the hypothesis of a "tubular phase" in the development of diabetic nephropathy. u-LFABP is seen to be independent predictor of microalbuminuria and death. Thus, u-LFABP may be used as an indicator of tubular damage early in the course of diabetes and therefore may find a place as a new tool in the prediction of diabetic nephropathy. However, further studies are needed for confirmation.

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References

1. Parving H-H, Mauer M, Ritz E. Diabetic nephropathy. In *Brenner & Rector's The Kidney*. 8th ed. Brenner BM, Ed. Boston, WB Saunders, 2008, p. 1265–1298
2. Soedamah-Muthu SS, Chaturvedi N, Witte DR, Stevens LK, Porta M, Fuller JH. Relationship between risk factors and mortality in type 1 diabetic patients in Europe: the , EURODIAB Prospective Complications Study (PCS). Diabetes Care 2008;31:1360–1366
3. Parving H-H, Østerby R, Ritz E. Diabetic nephropathy. In *The Kidney*. 6th ed. Brenner BM, Ed. Philadelphia, WB Saunders, 2000, p. 1731–1773
4. Maatman RG, van de Westerlo EM, van Kuppevelt TH, Veerkamp JH. Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney. Use of the reverse transcriptase polymerase chain reaction. Biochem J 1992;288(Pt. 1):285–290
5. Kamijo A, Sugaya T, Hikawa A, Okada M, Okumura F, Yamanouchi M, Honda A, Okabe M, Fujino T, Hirata Y, Omata M, Kaneko R, Fujii H, Fukamizu A, Kimura K. Urinary excretion of fatty acid-binding protein reflects stress overload on the proximal tubules. Am J Pathol 2004;165:1243–1255
6. Nielsen SE, Sugaya T, Tarnow L, Lajer M, Schjoedt KJ, Astrup AS, Baba T, Parving HH, Rossing P. Tubular and glomerular injury in diabetes and the impact of ACE inhibition. Diabetes Care 2009;32:1684–1688
7. Hovind P, Tarnow L, Rossing P, Jensen BR, Graae M, Torp I, Binder C, Parving HH. Predictors for the development of microalbuminuria and macroalbuminuria in patients with type 1 diabetes: inception cohort study. BMJ 2004;328:1105
8. Hovind P, Tarnow L, Rossing K, Rossing P, Eising S, Larsen N, Binder C, Parving HH. Decreasing incidence of severe diabetic microangiopathy in type 1 diabetes. Diabetes Care 2003;26:1258–1264
9. Nakamura T, Sugaya T, Kawagoe Y, Ueda Y, Osada S, Koide H. Effect of pitavastatin on urinary liver-type fatty acid-binding protein levels in patients with early diabetic nephropathy. Diabetes Care 2005; 28:2728–2732
10. Kamijo A, Sugaya T, Tanaka Y, Yasuda T, Kimura K. Urinary excretion of liver-type fatty acid binding protein (L-FABP) as a useful predictor of future renal in dysfunction in type 2 diabetic nephropathy. Abstract. presented at Renal Week 27 Oct–1 Nov 2009, San Diego, CA
11. Kamijo A, Sugaya T, Hikawa A, Yamanouchi M, Hirata Y, Ishimitsu T, Numabe A, Takagi M, Hayakawa H, Tabei F, Sugimoto T, Mise N, Kimura K. Clinical evaluation of urinary excretion of liver-type fatty acid-binding protein as a marker for the monitoring of chronic kidney disease: a multicenter trial. J Lab Clin Med 2005;145: 125–133
12. Sejdiu I, Torffvit O. Decreased urinary concentration of Tamm-Horsfall protein is associated with development of renal failure and cardiovascular death within 20 years in type 1 but not in type 2 diabetic patients. Scand J Urol Nephrol 2008;42:168–174
13. Maatman RG, van de Westerlo EM, van Kuppevelt TH, Veerkamp JH. Molecular identification of the liver- and the heart-type fatty acid-binding proteins in human and rat kidney. Use of the reverse transcriptase polymerase chain reaction. Biochem J 1992;288(Pt. 1):285–290
14. Sejdiu I, Torffvit O. Urinary excretion of fatty acid-binding protein reflects stress overload on the proximal tubules. Am J Pathol 2004;165:1243–1255
15. Yokoyama T, Kamijo-Ikemori A, Sugaya T, Hoshino S, Yasuda T, Kimura K. Urinary excretion of liver type fatty acid binding protein accurately reflects the degree of tubulointerstitial damage. Am J Pathol 2009;174:2096–2106
16. Glatz JF, van der Vusse GJ. Cellular fatty acid-binding proteins: their function and physiological significance. Prog Lipid Res 1996;35:243–282
17. Zimmerman AW, Veerkamp JH. Fatty-acid-binding proteins do not protect against induced cytotoxicity in a kidney cell model. Biochem J 2001;360:159–165