Urinary liver-type fatty acid-binding protein (u-LFABP) predicts progression to nephropathy in type 1 diabetic patients.

Running title: The tubular marker u-LFABP in type 1 diabetes

Authors: Stine Elkjaer Nielsen, M.D.¹, Takeshi Sugaya, M.D.,PhD ², Peter Hovind, M.D., DMSc.¹³, Tsuneharu Baba, M.D., PhD⁴, Hans-Henrik Parving, M.D. DMSc.⁵⁶, Peter Rossing, M.D. DMSc.¹

¹Steno Diabetes Center, Gentofte, Denmark
²Research Unit for Organ Regeneration, Riken Kobe Institute, Hyogo, Japan
³Department of Clinical Physiology and Nuclear Medicine, Glostrup Hospital, Copenhagen, Denmark
⁴Third department of Internal Medicine, School of Medicine, Fukushima Prefecture University, Fukushima, Japan
⁵Department of Medical Endocrinology, Rigshospitalet, University Hospital of Copenhagen, 2100 Copenhagen, Denmark
⁶Professor at Faculty of Health Science, Aarhus University, Denmark

Corresponding Author:
Stine E. Nielsen
M.D. Ph.D.student
e-mail: sene@steno.dk

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Background and aim: 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.

Methods: From an inception cohort of 277 patients, u-LFABP, adjusted for u-creatinine, (ELISA, CMIC ®) was measured in 24 hour urine in 165 normoalbuminuric patients 9.6± 3.5 (mean ±SD) years after onset of type 1 diabetes. Outcome: Development of persistent micro- or macroalbuminuria or death.

Results: Patients were followed for median (range): 18 (1-19) years and 39 progressed to microalbuminuria, and eight of those 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, Hba1c, systolic and diastolic blood pressure, albumin excretion rate, serum creatinine, smoking): hazard ratio (HR)(CI 95%): 2.3 (1.1-4.6) and log u-LFABP predicted mortality HR (95%CI): 3.0 (1.3-7.0) (adjusted). U-LFABP (above vs. below the median) predicted the development of macroalbuminuria HR (95% CI): 2.6 (1.2-5.4) (adjusted). As a continuous variable, u-LFABP tended to predict macroalbuminuria (HR 1.9, p=0.2), but numbers were small.

Conclusion: 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.
During the last decades the prevention and treatment of late complications in diabetes have improved dramatically. The focus on prevention of late diabetic complications has changed and now involves tight glycaemic 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. Additionally, 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 be potentially 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. First of all, this could help us to more accurately predict the patients at risk of developing diabetic nephropathy and secondly provide a better and possibly different treatment of 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; micro-/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 micro-/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. See figure 1 for design and flow chart. The patients were newly diagnosed with type 1 diabetes. They were treated according to guidelines described earlier(7) and were followed yearly with blood- and urine samples(8). Unfortunately, urines from before 1990 were lost, so in our analyses we included urine from 1990 and onward. Urine samples were available in 204 patients and out of these, 165 patients were normoalbuminuric, and in 2008 we decided to analyze their urine samples for u-LFABP. Urinary LFABP was measured in the first urine sample available after 1990(mean ±SD): 9.6 ± 3.5 years after onset of type 1 diabetes, and this was considered baseline in the present study (see figure 1). Hereafter, the patients were followed regarding endpoints for a median (range) of 18 (1-19) years. The primary outcomes in our study were time to development of micro-/macroalbuminuria or death, and were evaluated from the time of the patients’ first urine after 1990 and until 2008 or 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 hours was measured yearly in each patient. Persistent microalbuminuria and persistent macroalbuminuria were defined as a urinary albumin excretion rate between 30 and 300 mg/24 h and >300 mg/24 h, respectively, in at least two of three consecutive samples. All urine samples were collected as 24-hour urine.

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 rest. Smoking history was determined via questionnaire, and patients were classified as smokers if they were smoking more than one cigarette per day.

Urine samples were stored at -20˚C until u-LFABP analysis in 2009. U-LFABP was measured in a 2-step sandwich enzyme-linked immunosorbent assay(9) and adjusted for u-creatinine. The inter- and intra-assay variation was 6.8 and 8.2% respectively.

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

**Statistical analysis:** Data are mean (SD) for the normally distributed variables. Variables with skewed distribution are given as geometric mean (95% CI). Cumulative incidences of microalbuminuria and macroalbuminuria were calculated using Cox regression analyses. In table 1 differences between groups are analysed using ANOVA test.

A Cox model for each of the three endpoints; development of microalbuminuria, macroalbuminuria or death were analyzed in all 165 patients. Microalbuminuria was most frequent, but a surrogate endpoint and thus we separately analyzed the development of macroalbuminuria. If a patient was categorized as “micro-/macroalbuminuric” (two out of three samples), they would not be re-categorized if they later regressed in albuminuria. Patients were followed to 2008 or were censored at time of death. Patients who were lost for follow-up were counted in the analyses using the results of their last urine available. By using the National Register vital status were available in all patients by the end of 2008.

Cox regression model was used to analyse u-LFABP as an explanatory variable for the development of micro- and macroalbuminuria or death. Subsequently the model was adjusted for known risk factors: sex, age, HbA1c, systolic and diastolic blood pressure, urine albumin excretion rate, 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, HbA1c or UACR 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 for predicting microalbuminuria, subsequently we applied the same model to the other endpoints.

U-LFABP was reported as a categorical variable in figure 2, for the presentation of a Kaplan-Meier plot. ROC curves were calculated in SPSS assuming non-parametric distribution of parameters for standard error of area.

Statistical significance was assumed for p<0.05. Data were analyzed using SPSS 15.0 (SPPS, Chicago IL).

**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 figure 1 for flowchart).
Mean 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 endpoint 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 (95%CI): 27% (20-35), macroalbuminuria 6% (2-10) and death 17% (11-23).

Baseline characteristics of the patients, when divided into groups according to the later development of micro- and macroalbuminuria or persistent normoalbuminuria, can be seen in table 1. Patients who later developed micro- or macroalbuminuria had a higher systolic and diastolic blood pressure than the patients with persistent normoalbuminuria (p<0.05). Hba1c was progressively greater in the macro- and microalbuminuric group compared to the normoalbuminuric group(p=0.02). Urinary albumin excretion rate was also increased in the patients later developing micro- 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 to the persistent normoalbuminuric patients, however this was not statistically significant (p=0.17 and p=0.52).

In a Cox regression model (see table 2), baseline log u-LFABP levels predicted microalbuminuria, when adjusted for known risk factors (age, sex, Hba1c, systolic and diastolic blood pressure, urine albumin excretion, serum creatinine, smoking), the area under the curve increases only slightly; from 0.80 (0.71-0.89) to 0.81 (0.72-0.91).

As demonstrated in a Kaplan-Meier plot in figure 2, u-LFABP divided into quartiles, predicts the development of microalbuminuria (p=0.024). Hazard ratio from first to fourth quartile is = 5.8, p=0.004.

As a continuous variable, u-LFABP tended to predict the development of macroalbuminuria hazard ratio: 1.9, but this was not statistically significant (p=0.2), most likely because numbers were small (n=8).

When analysed as a categorical variable u-LFABP (above vs. below the median =10.9 (pg/ml)/(mg/dl creatinine)) predicted the development of macroalbuminuria: hazard ratio (95% CI): 2.6 (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; hazard ratio (95%CI): 3.0 (1.3-7.0) (adjusted).

**CONCLUSION**

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 (95%CI): 27% (20-35) and 6% (2-10) respectively.

Previously, we demonstrated that u-LFABP is increased already in the normoalbuminuric type 1 diabetic patients, indicating a “tubular phase” in the pathogenesis to diabetic kidney damage(10). However this was done in a cross-sectional study which in its design has some limitations, thus nothing can be concluded on...
the time perspective between elevated u-LFABP and progression to micro- 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 stage 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 u-albumin excretion in the normal range is also a strong predictor of progression to microalbuminuria. However our aim was to supplement u-albumin rather than replacing 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 at ASN 2009 Kamijo et al reported that u-LFABP predicts decrease in eGFR in type 2 diabetic patients with diabetic nephropathy(11). This supports our results and adds that u-LFABP is a promising marker in not only type 1, but also type 2 diabetic patients. In a study of non-diabetic patients with chronic kidney disease similar results have been found: patients with u-LFABP levels above the median deteriorated faster in kidney function than patients with u-LFABP below the median during one year of follow up(12). 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 uraemia in type 1, but not type 2 diabetic patients(13). This again supports the hypothesis that tubular markers could be added to albuminiuria 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 is most likely that effect of u-LFABP is mediated through the development of elevated urine albumin excretion. We found that patients who died had a significant higher incidence of micro-/macroalbuminuria than patients who survived during follow-up (41% vs 21%, p=0.027).

By studying renal biopsies with immunohistochemic staining, it has been shown that u-LFABP excretion is closely associated with structural and functional tubular kidney damage(14). This was confirmed in patients with chronic kidney disease including minimal change nephrotic syndrome, nephrosclerosis, lupus nephritis and diabetic nephropathy(15). 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(16). 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(10). This 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 hypothesised that LFABP is a protective protein(17), however previous studies have, to our knowledge, not been able to confirm this(18). U-LFABP as a continuous variable did not predict macroalbuminuria, it did when treated as a categorical variable. Due to 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 hour urine and
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The urine samples were stored at –20°C for approximately 18 years before analyses. Another limitation is the loss of urines before 1990 (see figure 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 micro-/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 endpoint.

In conclusion, we demonstrate that u-LFABP is elevated at an early stage, even before any clinical signs of glomerular damage is 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.

This indicates that 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 this needs to be confirmed in future studies.

DISCLOSURES:
T.Sugaya is the director and senior scientist of CMIC, the company that produced the kits for LFABP analysis. None of the other authors have conflicts of interest or financial disclosures of any relation to the present.

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Figure Legends

Figure 1: Design of the study

Figure 2: Kaplan Meier plot: Elevated baseline u-LFABP levels in 165 normoalbuminuric type 1 diabetic patients predict progression to microalbuminuria
REFERENCES


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 micro- or macroalbuminuria or persistent normoalbuminuria.

<table>
<thead>
<tr>
<th></th>
<th>Persistent normoalbuminuria</th>
<th>Only microalbuminuria</th>
<th>Macroalbuminuria</th>
<th>Progressors (micro+macro)</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>N (male/female)</td>
<td>62/64</td>
<td>22/9</td>
<td>6/2</td>
<td>28/11</td>
<td>0.05</td>
</tr>
<tr>
<td>Age (years)</td>
<td>37 (12)</td>
<td>41.7 (2.4)</td>
<td>43 (9)</td>
<td>41 (15)</td>
<td>0.18</td>
</tr>
<tr>
<td>Diabetes duration</td>
<td>8.6 (3.4)</td>
<td>8.4 (1.8)</td>
<td>12.1 (4.5)</td>
<td>9.0 (2.8)</td>
<td>0.05</td>
</tr>
<tr>
<td>Blood pressure systolic</td>
<td>122 (15)</td>
<td>128 (16)</td>
<td>138 (20)</td>
<td>130 (17)</td>
<td>0.02</td>
</tr>
<tr>
<td>Blood pressure diastolic</td>
<td>77 (8)</td>
<td>79 (9)</td>
<td>86 (14)</td>
<td>81 (10)</td>
<td>0.01</td>
</tr>
<tr>
<td>UAER (mg/24h)*</td>
<td>8 (7-9)</td>
<td>11 (8-14)</td>
<td>12 (7-20)</td>
<td>11 (9-14)</td>
<td>0.02</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>8.2 (1.1)</td>
<td>8.6 (1.7)</td>
<td>9.1 (0.9)</td>
<td>8.7 (1.6)</td>
<td>0.02</td>
</tr>
<tr>
<td>Serum creatinine (µmol/l)</td>
<td>72 (11)</td>
<td>69 (12)</td>
<td>70 (13)</td>
<td>69 (12)</td>
<td>0.48</td>
</tr>
<tr>
<td>U-LFABP/creat.*</td>
<td>9.6 (7.8-11.8)</td>
<td>13.4 (8.4-21.3)</td>
<td>12.6 (8.4-21)</td>
<td>13.2 (8.8-19.9)</td>
<td>0.35</td>
</tr>
</tbody>
</table>

Data are mean (SD), *=geometric mean (95 %CI), P= Overall difference between normo-, micro- and macroalbuminuria group compared with ANOVA test.
Microalbuminuria group does not include patients that later develop macroalbuminuria
UAER: Urine albumin excretion rate

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

<table>
<thead>
<tr>
<th></th>
<th>Odds Ratio</th>
<th>95% CI Lower limit</th>
<th>95% CI Upper limit</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sex (male)</td>
<td>4.19</td>
<td>1.62</td>
<td>10.87</td>
<td>0.003</td>
</tr>
<tr>
<td>Age (year)</td>
<td>1.02</td>
<td>0.99</td>
<td>1.05</td>
<td>0.274</td>
</tr>
<tr>
<td>Hba1c (%)</td>
<td>2.00</td>
<td>1.36</td>
<td>2.95</td>
<td>&lt;0.001</td>
</tr>
<tr>
<td>Blood Pressure Systolic</td>
<td>1.03</td>
<td>0.995</td>
<td>1.06</td>
<td>0.100</td>
</tr>
<tr>
<td>Blood Pressure Diastolic</td>
<td>0.99</td>
<td>0.94</td>
<td>1.04</td>
<td>0.758</td>
</tr>
<tr>
<td>Log (u-albumin(mg)/24hour)</td>
<td>12.36</td>
<td>2.58</td>
<td>59.32</td>
<td>0.002</td>
</tr>
<tr>
<td>Log (u-LFABP(ng/ml)/u-crea)</td>
<td>2.28</td>
<td>1.14</td>
<td>4.58</td>
<td>0.021</td>
</tr>
</tbody>
</table>

The analyses includes: sex, age, Hba1c, systolic and diastolic blood pressure, UAER, u-LFABP, s-creatinine (not shown) and smoking (not shown).
Figure 1: Design of the study

Year 1979-1984: Newly diagnosed DM1
277 patients

~Year 1990:
Urine samples not available
73 patients

~Year 1990:
Urine samples available
204 patients

~Year 1990:
Normoalbuminuria
165 patients

Follow-up until year 2008:
Persistent normoalb.
126 patients

Follow-up until year 2008:
Microalbuminuria
39 patients

Follow-up until year 2008:
Persistent macroalb.
8 patients

~Year 1990:
Microalbuminuria
32 patients

~Year 1990:
Macroalbuminuria
7 patients

BASELINE
Figure 2: Kaplan Meier plot: Elevated baseline u-LFABP levels in 165 normoalbuminuric type 1 diabetic patients predict progression to microalbuminuria

$p = 0.02$ for overall difference

Quartiles with limits: u-LFABP/creatinine: 4.7, 10.9 and 21.3 (pg/ml)/(mg/dl)