

## Tubular and glomerular injury in diabetes and the impact of ACE inhibition

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Submitted on 5 March 2009 and accepted 25 May 2009

Clinical trial reg. no. NCT00118976, clinicaltrials.gov

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*Objective:* We studied tubular and glomerular damage in type 1 diabetic patients by measuring u-LFABP (urinary liver-type fatty acid-binding protein) and albuminuria. Subsequently, we evaluated the effect of ACE inhibition on u-LFABP in patients with diabetic nephropathy.

*Research Design and Methods:* Caucasians with type 1 diabetes (T1D): 58 with normoalbuminuria (u-albumin<30mg/24h), 45 with persistent microalbuminuria (30-300 mg/24h) and 45 with persistent macroalbuminuria ( $\geq$ 300 mg/24h). A control group consisted of 57 healthy individuals. The groups were matched by gender and duration of diabetes. In addition U-LFABP were measured in 48 T1D patients with diabetic nephropathy in a randomized crossover trial consisting of 2 months of treatment with lisinopril 20, 40, and 60 mg once daily in random order.

*Results:* In the cross sectional study, the median levels (IQR) of u-LFABP ( $\mu$ g/g creatinine) were significantly higher in normoalbuminuric vs. control group (2.6 (1.3-4.1) vs. 1.9 (0.8-3.0),  $p=0.02$ ) and increased with increasing levels of albuminuria, microalbuminuric 4.2 (1.8-8.3), nephropathy group 71.2 (8.1-123.4),  $p<0.05$  for all comparisons. U-LFABP correlates with urinary albumin/creatinine ratio (UACR) ( $R^2=0.54$ ,  $p<0.001$ ).

In the intervention study, all doses of lisinopril significantly reduced urinary albumine excretion rate (UAER) and u-LFABP from baseline. The reductions (95%CI) in u-LFABP were 43%, 46%, and 40% with increasing doses of lisinopril (NS).

*Conclusion:* Early and progressive rise in tubulointerstitial damage as reflected by increased u-LFABP levels occurs in type 1 diabetic patients and is associated with albuminuria. Furthermore, ACE inhibition reduces the tubular and glomerular damage and dysfunction.

**D**iabetic nephropathy is a serious and common complication in diabetic patients. Although studies from selected centres suggest a declining incidence of diabetic nephropathy(1), still 30-40% of all patients with diabetes develop this complication (2). Diabetic nephropathy is associated with a higher risk of other complications such as cardiovascular disease, retinopathy, and neuropathy. It is the most common cause of end-stage renal failure in western countries. Therefore, it is of great interest to predict and prevent the development of diabetic nephropathy.

Persistent microalbuminuria is the established predictor of development of diabetic nephropathy and progressive renal insufficiency. However urinary albumin excretion rate, as an indicator of renal damage, has some limitations. Several patients with microalbuminuria do not progress to macroalbuminuri but continue having microalbuminuria (30%) or even regress to normoalbuminuria (15-30% after 7.5 years follow-up)(3).

As reviewed by Gilbert and Cooper, not only glomerular but also tubulointerstitial damage are important factors in the pathology of diabetic nephropathy (2,4).

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(5). By immunohistochemic staining of renal biopsies it has been shown that U-LFABP excretion is highly associated with structural and functional tubular kidney damage. This was confirmed in patients with chronic kidney disease including minimal change nephritic syndrome, nephrosclerosis, lupus nephritis and diabetic nephropathy(6). A previous study in CKD has shown that serum LFABP levels do not affect urinary LFABP level which suggests that there is no

transglomerular passage of LFABP in CKD and that the LFABP measured in urine primarily originates from tubular cells(7).

It has been hypothesised that LFABP is a protective protein, however previous studies have, to our knowledge, not been able to confirm this(8)

In patients with diabetic nephropathy a reduction in albuminuria is a predictor of renoprotection (9). It is suggested that the combination of the two parameters, albuminuria and u-LFABP, would be useful in monitoring chronic kidney disease more than either alone.

The relationship between albuminuria and u-LFABP has, to our knowledge, not been studied in type 1 diabetic patients. Furthermore it has not been studied if u-LFABP could be used to monitor the renoprotective effect on decline in GFR or in short term studies monitor the effect on urinary albumin excretion in type 1 diabetic patients with diabetic nephropathy.

The aim of this study was to investigate the levels of u-LFABP in a cross sectional study of type 1 diabetic patients with different levels of albuminuria and compare these with a non-diabetic control group. Additionally we aimed to explore the short term effect of increasing doses of the ACE inhibitor lisinopril on u-LFABP levels in patients with type 1 diabetes and diabetic nephropathy from a randomised, double-blind cross-over study.

## **RESEARCH DESIGN AND METHODS**

The cross sectional study was based on data from a cohort used to identify biomarkers of diabetic nephropathy by proteomic analyses(10). The population consisted of Caucasian patients with type 1 diabetes and different levels of albuminuria recruited from the out patient clinic at Steno Diabetes Center in 2004. Based on albumin excretion in 24 hour urine collections as part of the routine

care of the patients prior to the present study, patients were divided into three groups: 58 with normoalbuminuria (u-albumin excretion rate <30 mg/24h), 45 with persistent microalbuminuria (u-albumin excretion rate between 30-300 mg/24h in at least 2 out of three consecutive samples) and 45 with persistent macroalbuminuria (u-albumin excretion rate >300 mg/24h in at least 2 out of three consecutive samples). Control group consisted of 57 non-diabetic healthy individuals. The 24h urines were only used for assessing inclusion criteria. Groups were matched by gender and duration of diabetes.

Investigations were performed in the morning. Arterial blood pressure was measured three times with an appropriate cuff size following at least 10-min rest. Urinary albumin concentration was measured by an enzyme immunoassay from early morning spot urine collections and expressed as urinary albumin / creatinine ratio. Serum and urine creatinine concentration was assessed by a kinetic Jaffé method. Estimated GFR (eGFR) was calculated using the re-expressed 4-variable MDRD study equation: (eGFR=175 x p-creatinine<sup>-1.154</sup> x age<sup>-0.203</sup> x 1.212 [if black] x 0.742 [if female]) as defined previously (11) where eGFR is expressed as mL/min/1.73 m<sup>2</sup> and plasma creatinine as mg/dL. U-LFABP was measured in a 2-step sandwich enzyme-linked immunosorbent assay(12) and expressed as urinary LFABP/ creatinine ratio. The inter- and intra-assay variation were 6.8 and 8.2 % respectively.

Plasma samples were stored at -80C, and urine samples were stored at -20C until analysis.

The second study, was a randomised double-masked crossover trial performed in 2005 (13). Patients were treated in random order with lisinopril 20 mg, 40 mg and 60 mg, each period lasting 2 months. A total of 56 patients with type 1 diabetes, hypertension

(>135/85 mmHg) and diabetic nephropathy were randomised in the study.

The primary endpoint was changes in UAER (µg/24hours). Albuminuria was determined in three consecutive 24-hour urine collections completed immediately before the end of each treatment period. Among the secondary endpoints measured at the end of each treatment period were 24-hour ambulatory blood pressure, eGFR in one 24 h urine collection. U-LFABP was also measured in 24h urine (µg/24 hours). For safety reasons, blood pressure, plasma potassium, plasma sodium, and plasma creatinine were determined 3 weeks after the beginning of each treatment period. GFR (baseline) was measured at baseline after a single intravenous injection of 3.7 MBq <sup>51</sup>Cr-EDTA at 8:30 a.m. by determining the radioactivity in venous blood samples taken 180, 200, 220, and 240 minutes after injection. The results were standardized for 1.73 m<sup>2</sup> body surface area. Blood pressure was measured by a 24-hour ambulatory blood pressure (ABP) device (Takeda TM2421; A & D Medical, Tokyo, Japan).

The study was initiated by 2 months wash out period where all antihypertensive drugs were withdrawn, except for slow-release furosemid in individual doses to prevent fluid retention, hyperkalemia and control blood pressure. Thereafter patients were treated in random order with lisinopril 20 mg, 40 mg and 60 mg, each period lasting 2 months. Dietary intake of protein and salt was not restricted.

Both studies were performed according to the principles of the Declaration of Helsinki and the intervention study approved by the ethical committee of Copenhagen County. (clinicaltrial.gov: NCT00118976). Written informed consent was obtained from all patients.

**Statistical analysis:** Normally distributed variables are expressed as means (SD) (baseline characteristics) and means

(SE). U-LFABP and UACR is given as median (Inter quartile range; IQR). All comparisons between groups of normally or log normally distributed parameters were performed with a oneway ANOVA test, and ordinal data were compared using Kruskal Wallis test and Mann Whitneys test. Changes in variables between visits during the intervention study are expressed as mean differences with 95% CI. Comparisons of log u-LFABP between each treatment period were performed using linear mixed models. The adapted model was one with fixed effects of treatment level, visit and carry-over (i.e. treatment level in the previous period) and a random effect of person included to account for the person independencies in data. Linear regression analysis was used to analyze for correlations between the change from baseline in u-LFABP based on difference in log transformed values and changes in UAER, ABP and eGFR respectively.  $P < 0.05$  was considered significant (two-tailed test). Data were evaluated using SPSS version 14.0 (SPSS, Chicago, IL, USA).

## RESULTS

**Cross-sectional study:** The clinical characteristics are shown in table 1. The patients were well matched regarding the duration of diabetes and gender. Patients with normoalbuminuria and microalbuminuria were slightly older than controls and patients with macroalbuminuria.

We found a significant difference in systolic blood pressure between the groups ( $p=0.009$ ), due to a difference between controls and patients with microalbuminuria ( $p=0.017$ ) and macroalbuminuria ( $p=0.001$ ). Diastolic blood pressure was significantly higher in controls whereas there was no difference between diabetic groups. eGFR was significantly lower in the macroalbuminuric groups than all other groups.

The significant difference in serume creatinine was due to difference between the macroalbuminuria group and all other groups.

**U-LFABP and u-albumin excretion:** U-LFABP levels are shown in table 1 and illustrated in figure 1. Median levels (IQR) of u-LFABP ratios ( $\mu\text{g/g}$  creatinine) were significantly higher in the normoalbuminuric group versus control group: (2.6 (1.3-4.1) versus 1.9 (0.8-3.0),  $p=0.02$ ) and increased with increasing levels of albuminuria: microalbuminuric group: 4.2 (1.8-8.3), macroalbuminuria group: 71.2 (8.1-123.4),  $p<0.05$  for all comparisons.

U-LFABP correlates with urinary albumin/creatinine ratio (UACR) ( $R^2=0.54$ ,  $p < 0.001$ ) in combined group of all diabetic patients. In the normoalbuminuric group there was also a significant but weak association between UACR and U-LFABP ( $R^2=0.07$ ,  $p=0.04$ ).

In patients with macroalbuminuria U-LFABP levels correlated with urinary albumin/creatinine ratio ( $R^2 = 0.50$ ,  $p < 0.001$ ), and eGFR ( $R^2 = 0.34$   $p < 0.001$ ). When adjusted for eGFR, there was still a significant difference in u-LFABP for comparisons between patients with macroalbuminuria and the other groups ( $p < 0.01$ ).

There was a significant correlation between u-LFABP and systolic blood pressure ( $R^2=0.07$ ,  $p < 0.001$ ). However this may be explained by the association between u-LFABP and renal function as it disappeared after adjustment for eGFR and UACR. There were no significant associations between u-LFABP and gender, age, BMI, diastolic blood pressure, cholesterol, or  $\text{Hb}_{\text{A1c}}$ .

**Effect of ACE inhibition on u-LFABP: intervention study:** A total of 56 patients were randomized in the study of which 49 patients completed. One patient did not have u-LFABP measured at baseline, results are given for the remaining 48.

Age at baseline was (mean (SD)) 50 (10) years and the duration of diabetes was 33

(10) years. Baseline UAER was [geometric mean (95% CI)] 365 (240 to 554) mg/24 hours. Baseline GFR (CrEDTA) was 73 (28) ml/min/1.73m<sup>2</sup>.

All doses of lisinopril significantly reduced UAER, u-LFABP, and arterial blood pressure compared to baseline (table 2). At baseline, u-LFABP was 12.69 (3.88 – 49.82) µg/24h. Reductions from baseline in u-LFABP (95% CI) were 43% (15-62), 46% (19-64) and 40% (11-60) with increasing doses of lisinopril (no significant difference between doses). The reduction in u-LFABP was associated with the changes in 24-hour systolic ambulatory blood pressure ( $R^2=0.22$ ,  $p<0.01$ ) (similar for diastolic ABP) and UAER ( $R^2=0.38$ ,  $p<0.001$ ), but not with changes in eGFR. (Data given for change from baseline to 40 mg of lisinopril which gave the largest response, but the same was found for the other doses of lisinopril). The decline in u-LFABP was still significant when adjusted for the decline in UAER and for 24-hour systolic blood pressure ( $p=0.011$ ).

## CONCLUSION

In our cross-sectional study, we have shown that the marker of tubulointerstitial damage: u- liver fatty acid- binding protein (u-LFABP) is elevated in type 1 diabetic patients compared to non-diabetic healthy controls. Furthermore we have shown, that u-LFABP is further increased in type 1 diabetic patients with micro- and macroalbuminuria reflecting increased tubular damage with increasing levels of albuminuria. There were no significant correlations between u-LFABP and sex, age, or Hb<sub>a1c</sub>.

In our randomized, double masked crossover study, ACE inhibition with lisinopril reduced u-LFABP. There was no significant difference in effect between doses of lisinopril from 20-60 mg daily.

Until now, previous studies of u-LFABP in diabetes have been cross-sectional studies on u-LFABP in type 2 diabetic

patients. Suzuki et al(14) performed a cross-sectional study in 356 adult type 2 diabetic patients. They divided the patients into four groups: normo-, micro-, macroalbuminuric and renal failure, but no control group were included. They reported a significant association between the stage of diabetic nephropathy and u-LFABP although no significant difference between the normo- and microalbuminuric group was seen.

The results from our cross sectional study show that patients with normoalbuminuria and type 1 diabetes had higher u-LFABP than the healthy controls (table 1). The normoalbuminuric group had a significantly higher level of albuminuria than the healthy controls. But when adjusted for this, there was still a significant difference in u-LFABP between the two groups ( $p=0.014$ ). One possible explanation is that having diabetes elevates u-LFABP. However this is not likely as u-LFABP is not correlated with Hb<sub>a1c</sub>. Another hypothesis could be, that part of the patients in the normoalbuminuric group has higher levels of u-LFABP as a predictor of future development of microalbuminuria and diabetic nephropathy. The significant association between UACR and U-LFABP in the normoalbuminuric group also indicated this. However, to test this hypothesis we need prospective follow-up studies in normoalbuminuric type 1 diabetic patients.

We also saw that with increasing levels of albuminuria, from normo- to micro- and macroalbuminuria, u-LFABP is increasing. Part of this association can be explained by the transport with albumin of fatty acids to the proximal tubules. Here the fatty acids are absorbed into the proximal tubular cells, where LFABPs role is to transport the fatty acids to the mitochondria. Therefore, when albuminuria increases, the LFABP gene is upregulated and more LFABP is excreted into the urine(15). U-LFABP is also elevated independently of albuminuria because of increased tubular production due

to tubular hypoxia and oxidative stress(16), which is seen in diabetes(17). Our study is cross-sectional, and therefore we can not conclude on the time perspective between elevation in u-LFABP and development of nephropathy. However, from earlier studies in non-diabetic chronic renal disease, the potential of u-LFABP as an early predictor of nephropathy is supported. Kamijo et al(18) performed a multi-center observational trial in 48 patients with non-diabetic chronic kidney disease. Retrospectively, they divided the patients into progressors and non-progressors in their chronic kidney disease based on changes in creatinine clearance during 1 year of follow up. They found that u-LFABP had a higher sensitivity (94%) than u-albumin (69%) but a lower specificity (63%) than u-albumin (94%) in predicting the progression in chronic kidney disease.

U-LFABP is different from other suggested biomarkers, e.g.  $\alpha$ 1- and  $\beta$ 2-microglobulin: U-LFABP is produced in the tubular cells, whereas  $\alpha$ 1- and  $\beta$ 2-microglobulin is freely filtered through the glomerular basement membrane. During poor glycemic control, urinary excretion of  $\beta$ 2-microglobulin is increased (19), this is a result of lacking reabsorption in the damaged tubular cell (20,21).  $\beta$ 2 microglobulin is unstable at low pH causing an underestimate of the tubular damage(22).

In our randomized, crossover study, we saw that 2 months of ACE inhibition reduces u-LFABP with approximately 40%. There was no significant difference in decline in u-LFABP between doses of lisinopril, suggesting optimal effect with 20 mg lisinopril daily, which was in contrast to the increased decline in UAER when doses were increased from 20 to 40 mg lisinopril.

We observed that the decrease in u-LFABP is associated with a decrease in albuminuria, but the relatively weak association ( $R^2 = 0.38$ ,  $p < 0.001$ ) supports that the decline in u-LFABP is not only explained

by reduced albuminuria, and suggests, as mentioned earlier, that the reduction also reflects reduced tubular damage. The decrease in u-LFABP indicates that the tubular damage and upregulation of the u-LFABP gene are reversible.

Our finding is in accordance with earlier studies in type 2 diabetic patients (23) which showed a significant decrease in u-LFABP, when these were treated with angiotensin II receptor antagonist. Experimental studies in diabetic rats have shown that RAAS blockade reduces anti-apoptotic factors(24) and that oxidative stress in tubular cells is reduced and chronic hypoxia is corrected, independently of the blood pressure lowering effect(25) and thereby preserves tubular function.

In conclusion, early and progressive rise in tubulointerstitial damage as reflected by increased u-LFABP levels occurs in type 1 diabetic patients and is associated with albuminuria. Furthermore, ACE inhibition reduces the tubular and glomerular damage and dysfunction

Our studies indicate that u-LFABP is a new marker of tubular damage and a potential supplement to the glomerular damage marker albuminuria for prognosis, diagnosis and treatment of kidney injury, although further longitudinal studies are needed.

## ACKNOWLEDGEMENTS

We would like to thank B.R. Jensen, T.R. Juhl, B.V. Hansen, U.M. Smidt and L.Pietraszek for their help with collecting the data.

**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.

The data has been presented at EASD 2008 .

**Funding:** The studies were financed out of local funds and were not supported by the pharmaceutical industry.

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**Table 1:** Clinical data of control group and type 1 diabetic patients differentiated according to the level of albuminuria in cross sectional study.

	Control group	Normoalbuminuria	Microalbuminuria	Macroalbuminuria	p -value
N (male/female)	57 (37/20)	58 (30/28)	45 (24/21)	45 (27/18)	0.273
Age (years)*	51 (11.0)	56 (10.8)	54 (11.1)	49 (9.3)	0.004
Diabetes duration (years)*	-	37 (11)	35 (11)	34 (11)	0.411
Systolic BP (mmHg) *	132 (16)	138 (21)	142 (23)	145 (19)	0.009
Diastolic BP (mmHg) *	81 (11)	75 (11)	74 (12)	78 (10)	0.002
Hb <sub>A1c</sub> (%) *	5.5 (0.3)	8.2 (1.1)	8.8 (1.2)	8.8 (1.1)	<0.001
eGFR* (ml/min/1.73m <sup>2</sup> )	70.5 (9.6)	70.3 (10.3)	71.0 (13.3)	48.7 (19.2)	<0.001
Creatinine (µmol/l) ***	95 (73-146)	91 (69-121)	89 (70-143)	127 (84-144)	<0.001
UACR (mg/g) **	2 (1-5)	5 (3-9)	27 (11-68)	461 (173-1172)	-
U-LFABP (µg/g creatinine) **	1.9 (0.8-3.0)	2.6 (1.3-4.1)	4.2 (1.8-8.3)	71.2 (8.1-123.4)	<0.05

Presented as numbers where not specified. \* Mean (SD), \*\* Median (IQR), \*\*\*median (range)

“p-value”: refers to all over difference between groups (ANOVA)

Hb<sub>A1c</sub>; hemoglobin A1c, BP; blood pressure, UACR; Urine albumine creatinine ratio, eGFR; estimated glomerular filtration rate (MDRD)

**Table 2:** Laboratory data during treatment with Lisinopril 20, 40 and 60 mg in random order compared to baseline, in 48 type 1 diabetic patients with diabetic nephropathy.

	Baseline	Lisinopril		
		20 mg	40 mg	60 mg
24h systolic BP(mmHg), reduction from baseline	-	10 (6-14) <sup>1</sup>	13 (8-18) <sup>1,2</sup>	12 (8-17) <sup>1,2</sup>
24h diastolic BP (mmHg) , reduction from baseline	-	5 (3-7) <sup>1</sup>	7 (5-10) <sup>1,2</sup>	7 (5-10) <sup>1,2</sup>
Reduction in UAER compared to baseline (95% CI)	-	63% (55-69) <sup>1</sup>	71% (66-76) <sup>1</sup>	70% (64-75) <sup>1</sup>
eGFR (mL/min/1.73 m <sup>2</sup> )	75 (4)	69 (4) <sup>1</sup>	68 (4) <sup>1</sup>	67 (4) <sup>1</sup>
Hb <sub>A1c</sub> (%)	8.6 (0.1)	8.7 (0.2)	8.8 (0.2)	8.9 (0.1) <sup>1</sup>
P-potassium (mmol/L)	3.9 (0.1)	4.3 (0.1) <sup>1</sup>	4.4 (0.1) <sup>1</sup>	4.4 (0.1) <sup>1</sup>
U-LFABP reduction compared to baseline (95% CI)	-	43% (15-62) <sup>1</sup>	46% (19-64) <sup>1</sup>	40% (11-60) <sup>1</sup>

Data are mean (SE), <sup>1</sup>p<0.05 vs. baseline, <sup>2</sup>p<0.05 vs. 20 mg.

Friedman test for several related samples was used followed by paired samples t-test if significant.

**Figure 1:** u-LFABP in control group and three diabetic groups with different levels of albuminuria.

