DOI: 10.7860/JCDR/2019/38098.12525

Original Article

Biochemistry Section

Assessment of Urinary Liver-Type Fatty Acid Binding Protein (LFABP) Levels in Type 2 Diabetes Mellitus Patients with Nephropathy

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ABSTRACT

Introduction: Diabetic Nephropathy (DN) is a micro-vascular complication of Type 2 Diabetes Mellitus (T2DM). It is known that renal tubular damage plays an important role in DN. Liver-Type Fatty Acid Binding Protein (LFABP) is found in liver and proximal tubular cells of kidney and its levels are associated with the structural and functional changes of tubular cells of kidney.

Aim: The present study was carried out to estimate the levels of urinary LFABP and its potential role as a clinical biomarker for early diagnosis of nephropathy in T2DM patients with nephropathy.

Materials and Methods: A hospital based cross-sectional study was conducted on 84 subjects divided into 3 groups. Group 1: (n=28) healthy controls, Group 2: (n=28) T2DM patients without nephropathy and Group 3: (n=28) T2DM patients with nephropathy. Serum and urine creatinine were carried out by alkaline picrate Jaffe's kinetic method. Urine albumin was estimated by turbidometric method by using nephelometer. Urinary LFABP levels were measured by commercial available ELISA kit.

Results: In the present study, a statistically significant difference was found in urinary Albumin Creatinine Ratio (ACR) between the study groups I and III, II and III (p=<0.001). Also, a statistically significant difference was found between the levels of urinary LFABP in groups I and II, I and III (p=0.004 and p=<0.001 respectively). Significantly increased level of urinary LFABP was found in group III as compared to group II. There was a positive correlation observed between urinary LFABP levels with urinary ACR, which indicated that LFABP can predict kidney damage even before micro-albuminuria can be detected. Also a negative correlation was observed between urinary LFABP and estimated Glomerular Filtration Rate (eGFR).

Conclusion: The study suggests that the estimation of urinary LFABP could be used as a potential adjunct biomarker along with urinary ACR for early detection of DN and monitoring of progression of DN in clinical practice. This will enable the Institution for preventive strategies that could delay the onset of symptoms of full-blown DM and End Stage Renal Disease (ESRD).

Keywords: Albumin creatinine ratio, Diabetic nephropathy, End stage renal disease, Glomerular filtration rate

INTRODUCTION

DN is a micro-vascular complication of diabetes and the leading cause of ESRD worldwide [1]. The Indian Chronic Kidney Disease (CKD) registry confirms DN as the pre-eminent cause of CKD in India and reported that in 2012, 31.3% DN patients developed CKD in India [2]. Renal hypertrophy, deposition of Extra Cellular Matrix (ECM) in mesangial cells, thickening of glomerular basement membrane, and glomerulosclerosis are the major renal functional changes associated with progressive glomerular capillary occlusion, albuminuria and a progressive fall in GFR [3].

Despite many years of research, pathogenesis of DN is still not clear. DN was initially considered to be a glomerular pathology, but various studies suggested that one third of patients had minor or no glomerular changes but showed multiple tubulointerstitial lesions [4,5]. It is known that tubulointerstitial damage plays an important role in DN [6]. The extent of tubulointerstitial damage has been found to be correlated more with the prognosis of CKD than is the degree of glomerular injury [7].

LFABP is an intracellular carrier protein which is found in liver and proximal tubular cells of kidney [8,9]. It has been documented that increased levels of urinary LFABP is associated with the structural and functional changes in tubular cells of kidney and therefore, can be used as biomarker of renal tubular damage in DN [10]. The renal tubulointerstitial damage plays an important role in DN, in such cases assessment of LFABP as a marker of tubular damage could be beneficial along with estimation of albuminuria, as a marker of glomerular damage, to determine the complete status of the renal damage in DN [11]. Numerous studies had been carried out in various countries to predict the usefulness of urinary LFABP as a marker for early detection of DN [11,12]. However, very few studies on clinical significance of urinary LFABP in DN have been done in India so far [13]. Estimation of urinary ACR has been used for early detection of persistent micro-albuminuria in diabetes mellitus patients [14]. Albuminuria was considered to be an important marker for nephropathy but it does not show promising results sometimes [15]. Nielsen SE et al., reported that the levels of urinary LFABP are found higher in diabetic patients, even before they develop any glomerular injury. This indicates that tubular damage occurs at an early stage of diabetic kidney damage, even before the development of micro- and macro-albuminuria in DM patients [16]. Various newer tubular biomarkers are being extensively studied to detect the nephropathy at an earlier stage and progression to ESRD can be prevented. Hence, this present study was undertaken to estimate the urinary LFABP levels and compare it with the conventional marker of kidney damage i.e., micro-albuminuria measured by urinary ACR in normal healthy controls, T2DM patients and T2DM patients with nephropathy.

MATERIALS AND METHODS

Study Design

This was a hospital based cross-sectional, case control study conducted at Diabetic and Nephrology clinic at University College

of Medical Sciences and Guru Teg Bahadur Hospital, Delhi, India. Total 84 patients were enrolled in the study. The subjects were categorised into 3 groups. Group 1: (n=28) healthy controls, Group 2: (n=28) T2DM patients, Group 3: (n=28) T2DM patients with nephropathy. Type 2 diabetic patients with nephropathy and without nephropathy, within the age group of 30 to 60 years and duration of diabetes ≥5, visiting the hospital from December 2013 to April 2015 were studied. Age and sex matched healthy controls (group 1) were recruited from among attendants of patients and the staff persons of University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi. Diagnosis of T2DM was based upon American Diabetes Association (ADA) 2011 guidelines [17]. DN was diagnosed on the basis of persistent micro-albuminuria (ACR 30-299 mg/g creatinine) or overt albuminuria (ACR ≥300 mg/g creatinine) on two separate occasions (six weeks apart) and presence of DN was tested on the basis of National Kidney Foundation (NKF) 2012 guidelines [18]. Patient's fundus examination was done by direct ophthalmoscopy to look for evidence of diabetic retinopathy. Patients with diabetes mellitus due to Type 1 or secondary cause, presence of urinary or systemic infection, patients taking aspirin and systemic steroids were excluded from study. The study was approved by Institutional Ethics Committee-Human Research (IEC-HR) of University College of Medical Sciences and Guru Teg Bahadur Hospital, Delhi and written informed consent for participation in this study was taken from all patients.

Estimation of Biochemical Parameters

Five-millilitre blood sample was collected for biochemical analysis. Blood was centrifuged at 3500 RPM for 15 minutes for plasma and serum separation. Plasma glucose level was measured by Glucose Oxidase-Peroxidase (GOD-POD) method and quantified spectrophotometrically at 500 nm [19]. Glycated Haemoglobin (HbA1c) was estimated by micro-column based technique and quantified spectrophotometrically at 500 nm [20]. Haemoglobin was estimated by cyanmethaemoglobin method and blood urea was estimated by Diacetyl Monoxime (DAM) method [21-22]. Plasma glucose, blood urea, serum creatinine and haemoglobin were processed on the same day. However, samples for glycated haemoglobin was stored at 2-8°C in refrigerator and processed within a week.

Estimation of Urinary ACR and GFR

Morning spot urine samples were collected for and processed on the same day to determine urine albumin and urine creatinine levels. Serum and urine creatinine were carried out by alkaline picrate Jaffe's kinetic method [23]. Picric acid in the presence of sodium hydroxide, an alkaline picrate complex is formed. This complex interacts with creatinine and forms an orange colour complex and intensity of this complex is directly proportional to the concentration of creatinine. The intensity was measured as Optical Density (OD) at 530 nm using multimode reader (Synergy H1 Hybrid Reader, BioTek). Urine albumin was estimated by nephelometry method by using nephelometer (Nephstar®, Goldsite, USA). The sensitivity limit was 10 mg/L. Albumin /creatinine ratio was expressed in mg/g creatinine. Estimated Glomerular Filtration Rate (eGFR) was calculated by Modification of Diet in Renal Disease (MDRD) equation [24].

MDRD Equation

$eGFR\ (mL/\ min) = 186 \times (Plasma\ creatinine\ in\ mg/\ dL)^{-1.154} \times (Age\ in\ years)^{-0.203} \times (0.742\ if\ female) \times (1.212\ if\ the\ patient\ is\ black)$.

Estimation of Urinary LFABP Levels

Urinary LFABP levels were measured by commercially available ELISA kit (CMIC Holdings Co. Ltd., Tokyo, Japan).

STATISTICAL ANALYSIS

Statistical analysis was carried out by the SPSS version 20.0. Data were expressed as mean±SD, median, (IQR) or percentage (%) as applicable. Differences between mean value of demographic and biochemical parameters amongst groups were compared by one-way ANOVA followed by post-hoc Tukey's test or Kruskal-Wallis test used for non-parametric data. Multivariate analysis was done using Bonferroni adjustment. Correlation was analysed by using Pearson's correlation coefficient. The $p<0.05$ was considered as the level of significance.

RESULTS

Demographic Characteristics and Biochemical Parameters of the Study Subjects

Various demographic and biochemical characteristics of the participants were studied [Table/Fig-1]. A statistically significant difference was found in SBP, DBP, FBG, PPBG levels when group II and III were compared with group I (healthy control). Also significant difference was observed for HbA1c values between group II and III (p-value-0.001). Patients in group III had significantly higher blood urea and serum creatinine levels when compared with group I or group II (p-value=0.001). A significant difference in mean value of eGFR was observed among groups (p-value=<0.001). A statistically significant difference found in the levels of urinary ACR between the study groups I and III, II and III, however, no statistically significant difference was observed between group I and II.

Urinary LFABP Levels in the Study Subjects

In the present study, urinary LFABP levels were significantly elevated in group II and III patients when compared to control group I. Urinary LFABP levels were found to be significantly higher in diabetic patients having micro- and macro-albuminuria (group III) in comparison to group II patients, indicating increased tubular damage with increasing levels of albuminuria (p-value=<0.001) [Table/Fig-2].

Correlation between Urinary LFABP and Other Variables

There was a statistical significant positive correlation found between urinary LFABP levels and duration of diabetes, serum creatinine and urinary ACR. A combination of urinary ACR and urinary LFABP could be a good marker for early diagnosis of DN. Correlation analysis also showed that urinary LFABP levels were negatively correlated with eGFR and this correlation was significant (p-value-0.000; r-value-0.395) [Table/Fig-3]. This correlation between urinary LFABP and eGFR supports the use of urinary LFABP as a marker of degree of renal damage as estimated by GFR.

DISCUSSION

Patients with DN are at a high risk for progression to the ESRD. Due to the asymptomatic nature in the early course of the disease, DN is frequently not detected until late stages, resulting in lost opportunities for prevention and treatment. Progression of renal failure or other adverse outcomes could be prevented or delayed through the early detection of DN. Urinary ACR is a commonly used marker to predict DN at an early stage. However, multiple studies have shown that micro-albuminuria (30-300 mg albumin/g creatinine) is not a specific marker for development of DN in type 2 diabetes as many patients who have micro-albuminuria at one point of time may not have it when measured later therefore; it is a poor predictor of the development of DN [25]. Various new markers are being identified which can detect nephropathy at an earlier stage. Neutrophil Gelatinase-Associated Lipocalin (NGAL), LFABP, Kidney Injury Molecule-1 (KIM-1), Interleukin-18 (IL-18) and TGF-β1 are few of the newer markers, increasingly studied for their potential as newer markers for diagnosis of nephropathy [26].

Parameters	Group I (n=28)	Group II (n=28)	Group III (n=28)	$p-value	#p-value
Male/Female	14/14	14/14	15/13	-	-
Age (years)	47.04±7.35	49.46±8.42	47.50±8.69	0.50	I and II=0.970 I and III=0.511 II and III=0.622
SBP (mmHg)	128.78±13.39	129.89±16.12	139.89±13.4	0.008*	I and II=0.950 I and III=0.013* II and III=0.029*
DBP (mmHg)	77.39±10.98	82.4±10.14	84.67±10.60	0.036*	I and II=0.189 I and III=0.031* II and III=0.700
FBG (mg/dL)	88.71±5.46	148.93±73.73	141.0±35.42	<0.001*	I and II=<0.001* I and III=0.001* II and III=0.832
PPBG (mg/dL)	129.0±12.18	215.21±75.87	195.91±53.11	<0.001*	I and II=<0.001* I and III=<0.001* II and III=0.425
Haemoglobin (g/dL)	12.30±1.46	12.35±1.66	11.27±1.94	0.03*	I and II=0.99 I and III=0.062 II and III=0.052
HbA1c (%)	-	7.71±0.95	10.36±1.80	0.001*	II and III=0.001*
Blood urea (mg/dL)	31.75±5.59	28.93±10.17	48.07±12.97	0.001*	I and II=0.54 I and III=0.001* II and III=0.001*
Serum creatinine (mg/dL)	0.85±0.12	0.89±0.17	1.45±0.27	0.001*	I and II=0.70 I and III=0.001* II and III=0.001*
eGFR (mL/min/1.73^2)	88.57±17.09	83.93±19.01	47.04±11.09	<0.001*	I and II=0.52 I and III=<0.001* II and III=<0.001*
Urinary ACR (mg/g creatinine)	10.51 (7.75-15.12)	16.06 (9.51-18.67)	274.75 (92.78-435.74)	<0.001*	I and II=0.348 I and III=<0.001* II and III=<0.001*

[Table/Fig-1]: Demographic characteristics and biochemical parameters in the study subjects.
Values are expressed as Mean±SD and Median (IQR). p-value is significant at p<0.05. *significant value, $One way ANOVA/ Kruskal Wallis Test; #Tukey's test/Bonferroni adjustment. SBP: Systolic blood pressure; DBP: Diastolic blood pressure; FBG: Fasting blood glucose; PPBG: Post-prandial blood glucose; eGFR: Estimated glomerular filtration rate and urinary; ACR: Urinary albumin creatinine ratio

Parameter	Group I (n=28)	Group II (n=28)	Group III (n=28)	$p-value	#p-value
Urinary LFABP (µg/g creatinine)	2.89 (2.16-5.44)	7.50 (4.91-28.25)	54.50 (10.87-97.53)	<0.001*	I and II=0.004* I and III=<0.001* II and III=0.005*

[Table/Fig-2]: Urinary LFABP levels of the study subjects.
Data are presented as Median (IQR), p-value is significant at p<0.05. *significant values, $Kruskal Wallis Test; #Bonferroni adjustment, LFABP: Liver fatty acid binding protein

Variables	Correlation coefficient (r)	p-value
Duration of diabetes (years)	0.269	0.04*
Serum creatinine (mg/dL)	0.230	<0.001*
eGFR (mL/min/1.73^2)	-0.395	<0.001*
Urinary ACR (mg/g creatinine)	0.559	<0.001*

[Table/Fig-3]: Correlation between urinary LFABP and other study variables.
Correlation is significant at p<0.05, *significant value, eGFR: Estimated glomerular filtration rate and urinary; ACR: Urinary albumin creatinine ratio

Previous reports have suggested that in DN, both glomerular damage and tubulointerstitial damage can lead to end-stage renal failure [27]. Urinary albumin reflects glomerular damage while, urinary LFABP reflects tubulointerstitial damage. The changes in urinary LFABP were significantly correlated with the progression of renal disease than those in urinary albumin, suggesting that urinary LFABP is more sensitive than urinary albumin in monitoring the progression of CKD [28]. However, several studies have shown that in addition to urinary albumin, urinary LFABP can be used as a potential clinical marker for identifying the patients who are likely to experience deterioration of renal function in DN patients [7,13]. In the Indian context, very few studies have reported the significance of LFABP levels in type 2 DM patients with nephropathy. Viswanathan V et al., evaluated the levels of urinary LFABP at different stages of DN and observed its correlation with other clinical parameters in South Indian patients with T2DM. They reported that urinary LFABP levels were increased in patients with reduced eGFR and showed a positive correlation with systolic blood pressure and protein to creatinine ratio in all the study subjects [13].

Our results are in accordance with the results reported by Suzuki K et al. They reported a significant association between the stage of DN and urinary LFABP, although no significant difference was found between the normo-albuminuric and micro-albuminuric groups in their study [29]. Kamijo-Ikemori A et al., also reported that urinary LFABP levels were progressively increased in subjects with normo-, micro- or macro-albuminuria, which further increased in patients with ESRD [12]. However, results of our study are in contrast with Abbate M et al., results states that tubular damage in DN may be a result of the tubulo-toxic effect of albumin and other proteins that are leaked into the tubular lumen [30]. But this tubulotoxic effect theory cannot explain our findings since urinary LFABP levels were found to be increased even in the normo-albuminuric diabetic patients compared to non-diabetic control subjects and Albuminuria (ACR) in these normo-albuminuric diabetic patients (group II) was comparable with albuminuria in non-diabetic control subjects (group I).

Our results were similar to results of Kamijo-Ikemori A et al., who showed that as eGFR progressively decreases from normo-, micro-, macro-albuminuria and ultimately end stage renal failure while, urinary LFABP level increases respectively [12]. The results of the present study are also in contrast with the result of Chou KM et al., who showed that tubular markers, such as NGAL and urinary LFABP, may not be predictive factors associated with GFR decline in T2DM patients [31]. The present study also showed

a positive correlation between urinary LFABP levels and urinary ACR which was statistically significant. Various studies have reported a positive correlation between LFABP levels and urinary ACR [29,32]. However, in the present study, there was a positive correlation between urinary LFABP and urinary ACR levels in diabetic patients with normo-albuminuria (group II) and diabetic patients with micro- and macro-albuminuria (group III) only, which was not statistically significant.

LIMITATION

In the present study, sample size was small. Other markers of tubular injury were not measured. Further research in a large-sized multicentre study is needed to validate our findings.

CONCLUSION

Urinary LFABP can reflect the patho-physiological condition of DN and the levels of urinary LFABP can help determine the severity of DN at earlier stages as compared to albuminuria. In clinical practice, in addition to urinary albumin, the measurement of urinary LFABP have emerged as a potential biomarker for early detection, assessment of severity of DN and monitoring of progression of DN patients.

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FINANCIAL OR OTHER COMPETING INTERESTS: None.

Date of Submission: **Jul 14, 2018**
Date of Peer Review: **Sep 08, 2018**
Date of Acceptance: **Nov 29, 2018**
Date of Publishing: **Jan 01, 2019**