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Predictive Roles of Urinary Liver Type - Fatty Acid-Binding Protein and N-Acetyl-β-D-Glucosaminidase for Progression of Diabetic Nephropathy in Type 2 Diabetic Patients

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ABSTRACT

Background: diabetes mellitus (DM) is a common metabolic disorder characterized by chronic hyperglycemia leading to significant morbidity and mortality as a result of the development of chronic macrovascular and microvascular complications. The onset of type 2 diabetes mellitus (T2DM) is often silent and insidious and this accounts for the relatively high prevalence of complications at initial presentation**.**

Objective: The aim of the current study was to evaluate the urinary level of liver type-fatty acid binding protein (u-L-FABP) as a proximal tubular damage biomarker in prediction or early detection of Diabetic nephropathy (DN) and whether its levels parallel the severity of kidney disease in type 2 diabetic patients, as assessed by the degree of albuminuria and other biochemical indices of renal dysfunction (e-GFR, serum creatinine and urea).

Patients and Methods: the study was conducted on 69 diabetic patients and 20 age and sex- matched apparently healthy control subjects. All patients included in this study were recruited from the inpatient and outpatient Endocrinology Clinic, Al-Zahraa University Hospital, between October 2016 and April 2018. The enrolled patients included 37 women and 32 men with age ranged from 40 to 67. They were diagnosed as having type 2 diabetes mellitus (T2DM)**.**

Results: Statistical analysis of results presumed that u-L-FABP levels >37.2 and 92.2 ng/L were the optimum cutoff levels to discriminate micro- and macroalbuminuric diabetic patients from controls with 90% and 100% diagnostic specificity and 96% and 100% accuracy, respectively. In addition, u-L-FABP levels > 28.5 and >386.1 ng/L, were the optimum cutoff values that predict the progression of microalbuminuria and macroalbuminuria with 100% diagnostic sensitivity and 87.9% and 99.7% accuracy, respectively.

Conclusion: the use of u-L-FABP as a specific proximal tubular damage biomarker alone, or together with microalbumin, is beneficial for early diagnosis and monitoring of DN, compared to u- N-Acetyl-β-D-Glucosaminidase (NAG) excretion.

Keywords: Urinary Liver- Fatty Acid-Binding, N-Acetyl-β-D-Glucosaminidase, Diabetic Nephropathy

INTRODUCTION

Diabetic nephropathy (DN) is one of the most clinically important microvascular complications of diabetes and is a leading cause to end-stage renal disease and kidney failure. It occurs in 20% to 40% of patients with type 2 diabetes [(1)] and has a strong association with other micro-and macrovascular diabetic complications with an increase in all-cause mortality [(2)]**.**

Therefore, early diagnostic markers for predicting and monitoring the progression of DN are needed to enable the timely administration of the most appropriate protective treatments [(3)]**.**

The evaluation of progression of DN is based on the degree of albuminuria and/or deterioration of renal function tests. These tests are not sensitive enough to detect early diabetic-kidney disease and so, they are used with some limitations [(4)]**.**

The condition is thus demanding the use of such sensitive biomarkers that can reflect the onset of DN at an early reversible stage to prevent the long-term devastating outcomes of renal loss in diabetics [(5)]**.**

A significant number of urinary biomarkers have been identified to reflect pathophysiology of DN [(6)]**.** In addition, urinary excretion of tubular damage markers has been found to be a non-invasive and sensitive measure for the evaluation of renal involvement in diabetes [(7)]**.**

N-acetyl-β-D-glucosaminidase (NAG) is a 130-140 KD hydrolytic enzyme, found in high concentration in lysosomes of proximal tubule epithelial cells and is involved in intracellular degradation of glycolipids and glycoproteins and also mucopolysaccharide and glycoprotein metabolism of tubular basement membrane [(8)]**.**

Because of its high molecular weight, plasma NAG cannot be filtered through the glomerulus and its increase in urine is caused exclusively by its secretion from the proximal tubular cell lysosomes because of increased lysosomal activity or upon injury affecting the tubular basement membrane [(9)]**.**

This indicates that in proteinuric glomerular diseases, the increased NAG excretion can occure secondary to increased uptake of high filtered proteins, even prior to microalbumin loss. with increasing of cellular albumin load, excessive leakage of the enzyme into urine occurs from damaged tubular cells, assuming that subclinical tubular dysfunction might develop earlier than glomerular [(9)]**.**

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In addition, liver-type fatty acid binding protein or fatty acid binding protein 1 (L-FABP) is 14 KD small intracellular cytoplasmic carrier protein primarily found in liver cells but was also found to be expressed in high concentration in the proximal tubule cells of the human kidneys [(10)]. It has a key role in binding and trafficking of fatty acids particularly long chain fatty acids across the cytosol to various cellular organelles to exert several putative functions [(11)].

Under normal physiological conditions, L-FABP derived from the liver is released into circulation, filtered through the glomeruli and reabsorbed into the proximal tubules. In renal disease however, tubulointerstitial damage reduces proximal tubular reabsorption of L-FABP and this leads to increased level of urinary L-FABP (u-L-FABP). These findings suggested that serum L-FABP levels do not influence urinary excretion of L-FABP which instead, is mostly determined by proximal tubule cell injury [(12)].

In addition, it has shown that excessive reabsorption of free fatty acids into the proximal tubules as in DM, induces tubulointerstitial damage [(13)]. Moreover, L-FABP gene expression in the kidney was found to be up-regulated with increasing L-FABP cellular levels and urinary excretion in stress conditions such as tubular ischemia, tubular stretch, protein overload, hypertension and hyperglycemia [(14)].

The aim of the current study was to evaluate the urinary level of liver type-fatty acid binding protein (u-L-FABP) as a proximal tubular damage biomarker in prediction or early detection of DN and whether its levels parallel the severity of kidney disease in type 2 diabetic patients, as assessed by the degree of albuminuria and other biochemical indices of renal dysfunction (e-GFR, serum creatinine and urea).

SUBJECTS AND METHODS

This study included a total of 69 diabetic patients diagnosed as having type 2 diabetes mellitus (T2DM) according to the diagnostic criteria of the **American Diabetes Association**[(15)] and 20 age and sex- matched apparently healthy control subjects, attending at inpatient and outpatient Endocrinology Clinic, Al-Zahraa University Hospital. This study was conducted between October 2016 and April 2018. Written informed consent of all the subjects was obtained.

Ethical approval:

The study protocol was approved by the Ethics Committees of the Clinical Pathology Department and Faculty of Medicine Administration, Al-Azhar University.

The enrolled patients included 37 females and 32 males with age ranged from 40 to 67.

Inclusion Criteria:

Patients free of systemic and local diseases other than diabetes mellitus or DN.

Exclusion Criteria:

Patients with liver and kidney diseases other than DN, including chronic renal failure, renal surgery, hemodialysis or transplantation, other endocrine disorders, malignancies, rheumatological diseases, lung and GIT trouble, hypertension, intake of nephrotoxic drugs, pregnancy and lactation, as well as those with positive results of urine dipsticks indicating urinary tract infection and/or hematuria, were excluded from the study. Female patients during menstruation were also excluded.

The age and sex- matched controls included 20 apparently healthy individuals 11 women and 9 men with a mean age of 50.90±6.36. They were selected with no history of diabetes mellitus or other exclusion criteria.

The included subjects were categorized according to the results of urinary albumin-creatinine ratio (UACR/ACR) into three subgroups; **Group I (**normoalbuminuric**)** consisted of 30 patients with ACR < 30 mg/g creatinine, **Group II (**microalbuminuric**)** consisted of 25 patients with ACR 30-300 mg/g creatinine and **Group III (**macroalbuminuric**)** consisted of 14 patients with ACR >300 mg/g creatinine.

All of the following was done to the enrolled subjects: full history taking, full clinical examination and laboratory investigations including: renal function tests (serum urea, creatinine, e-GFR, urinary albumin and ACR); Urine examination, using Medi-Test reagent strips (combi 10); liver enzymes (ALT and AST); lipid profile (total cholesterol, triglycerides, HDL-cholesterol and LDL-cholesterol and fasting plasma glucose level. All of them were done on cobas 311 auto-analyzer using Roch reagents kits. Determination glycated hemoglobin A1c (HbA1c%), was done using Bio-Rad auto-analyzer based on the principles of ion-exchange high performance liquid chromatography (HPLC). Estimation of urinary NAG and L-FABP was done by using ELISA kit.

Patient and control sterile urine specimens were collected. Every specimen was examined immediately by dipsticks, using Medi-test reagent strips (combi10) for urinalysis,

Sampling:

Second morning urine samples for:

• Immediate urine analysis by dipsticks based on comparison of the test paper attached to a plastic strip with the color chart blocks printed on the vial label. Test result may provide information regarding the status of carbohydrate metabolism, kidney and liver functions, acid-base balance, hematuria and urinary tract infection [(15)].

• Determination of ACR (preserved specimens at 2-8 °C for up to 7 days).
• Determination of urinary NAG and L-FABP (centrifuged samples for 20 minutes at a speed of 3000 rpm and the supernatant was further aliquoted into 2 Eppendorf tubes and kept refrigerated at -20°Cand kept frozen until assayed).

Venous blood samples (about 5 ml) after an overnight fasting for:

• immediate determination of biochemical parameters using tube containing gel for serum separation.
• Estimation of HbA_1c% using K3 EDTA tube and refrigerated at 2-8ºC to be analyzed within one week.

Statistical analysis

Data were collected, tabulated and analyzed using statistical software package SPSS (Version.20; Chicago, USA). Data are presented as Mean ± SD, median and IQR, number or percentage as appropriate. Qualitative data were analyzed using chi-square test. Parametric numerical values were analyzed using ANOVA and Post-hoc tests. Non Parametric numerical values were analyzed using. Kruskall Wallis and Mann-Whitney U tests. correlations between different parameters were analyzed using Spearman's rank correlation coefficient. Pvalue<0.05 were considered significant.

RESULTS

a- Descriptive statistics of demographic data and different studied parameters in serum and urine of all diabetic subgroups and control group are illustrated in tables (1 and 2).

b- Comparative analysis revealed highly significant increase in the duration of diabetes between patients with increasing levels of albuminuria ($p<0.001$) particularly in macroalbuminuric ($p<0.05$) (Table 3). In addition, there were significant increases in glycemic parameters (FPG and HbA_1c) in comparison to controls, especially among microalbnminuric patients ($P<0.05$). Moreover, there was a significant decline in kidney function as assessed by creatinine and e-GFR in both micro- and macroalbuminuric groups compared to control and normoalbuminuric groups ($P<0.05$) with further significant decline in macroalbuminuric group III than microalbuminuric group II ($P<0.05$). Also, lipid profile showed significant alteration among diabetic subgroups with significant increases in serum TC in comparison to controls ($P<0.05$) and reduction of HDL-C in macroalbuminuric group in comparison to other groups ($P<0.05$). While, LDL-C was significantly increased in micro- and macroalbuminuric groups as compared to controls and normoalbuminuric group ($P<0.05$). Diabetic subgroups also showed significant increases in TG levels in comparison to controls with more significant increase in micro- and macroalbuminuric groups and further increase among patients with macroalbuminuria (Table 4).

Statistically significant positive correlations were found between u-NAG (u/L) and u-L-FABP (ng/L) in all diabetic subgroups (I, II, III) particularly patient group II (microalbuminuric) ($P<0.001$). It was also detected in patients group including all diabetic cases ($P<0.001$) and when both markers are expressed in relation to urine creatinine concentration ($P<0.001$).

However, the correlation analysis of both tubular markers with other studied parameters in different diabetic subgroups did not revealed any significant associations (data not shown).

Nevertheless, when all diabetic cases are included as a whole, u-NAG (u/L) and u-L-FABP (ng/L) showed significant positive correlation with the duration of diabetes ($p<0.001$ and <0.05, respectively). In addition, u-L-FABP (ng/L) was significantly associated with parameters of renal dysfunction. This was indicated by the highly significant positive correlations between u-L-FABP and both serum urea ($p=0.006$) and creatinine ($p<0.001$), as well as the significant negative correlation with e-GFR ($p=0.001$) (Table 5 and Figures 1 and 2). U-L-FABP also showed a significant positive correlation with ACR when it is expressed in relation to urine creatinine ($p<0.001$) (Table 7). On the other hand, ACR was the only significant variable that directly correlated with u-NAG excretion expressed in relation to urine creatinine ($p=0.002$) (Table 7 and figure 4).

When evaluating the diagnostic performance of both tubular damage biomarkers in discriminating diabetic subgroups and control group and also, in differentiating between diabetic patients with increasing levels of albuminuria, (efficacy)

ROC curve analysis revealed non-significant discriminative abilities for u-NAG. On the other hand, u-L-FABP showed significant discriminative power to differentiate between all studied groups ($p<0.05$) except between normoalbuminuric patient group (group I) and control group (Figures 1-4).

Statistical analysis of our results presumed that u-L-FABP levels>37.2 and 92.2 ng/L were the optimum cutoff levels to discriminate micro- and macroalbuminuric diabetic patients from controls with 90% and 100% diagnostic specificity and 96% and 100% accuracy, respectively. In addition, u-L-FABP levels > 28.5 and >386.1 ng/L, were the optimum cutoff values that predict the progression of microalbuminuria and macroalbuminuria with 100% diagnostic sensitivity and 87.9% and 99.7% accuracy, respectively.

Table (1): Descriptive statistics of the demographic data and serum parameters in all studied groups.

Demographic Data	Control Group (N=20)	Group I normoalbuminuric (N=30)	Group II microalbuminuric (N=25)	Group III macroalbuminuric (N=14)
Age (years)				
Range	40-60	40-67	43-60	40-63
Mean±SD	50.90±6.36	50.63±6.63	51.68±3.85	50.93±7.26
Sex				
Female	11 (55%)	16 (53.3%)	14 (56%)	7 (50%)
Male	9 (45%)	14 (46.7%)	11 (44%)	7 (50%)
Duration (years)				
Range	-	1-15	7-20	14-30
Mean±SD	-	7.60±3.94	12.00±3.49	19.64±3.86
Glycemic parameters:				
FPG (mg/dL)				
Mean±SD	76.50±4.72	174.14±8.94	274.28±6.14	199.50±8.88
HbA_1c %				
Range	4.5-5.6	6.9-10.5	7.9-15.1	8.1-12.2
Mean±SD	5.22±0.38	8.78±1.91	10.06±1.24	9.14±0.71
Kidney function tests:				
Urea (mg/dL)				
Range	15-27	18-41	20-62	22-75
Mean±SD	24.50±3.35	26.32±4.86	44.40±8.73	56.21±10.19
Creatinine (mg/dL)				
Range	0.4-1.1	0.5-1.3	0.8-1.98	0.9-2.62
Mean±SD	0.75±0.09	0.78±0.11	1.56±0.24	2.14±0.64
e-GFR (ml/min/1.73m^2)				
Range	75-114	74-106	55-79.83	35-64
Mean±SD	97.33±8.1	94.5±6.3	68.75±5.2	55.28±2.65
Liver enzymes:				
ALT (U/L)				
Mean ± SD	16.10±2.64	16.90±3.86	19.60±3.29	20.64±3.87
AST (U/L)				
Mean ± SD	17.20±3.69	18.13±3.26	18.64±3.26	19.29±3.65
Lipid profile:				
TG (mg/dL)				
Mean ± SD	105.05±9.94	138.10±4.52	163.57±6.85	222.68±7.19
TC (mg/dL)				
Range	120-193	92-273	116-299	131-334
Mean ± SD	165.05±19.92	203.90±45.44	211.04±44.42	215.50±47.66
HDL-C (mg/dL)				
Mean ± SD	43.68±5.07	42.96±9.12	40.39±7.80	37.40±5.85
LDL-C (mg/dL)				
Mean ± SD	101.40±12.75	101.61±3.64	129.32±4.20	135.51±5.99

Table (2): Descriptive statistics of urine parameters in all studied groups.

Studied Parameters	Control Group (N=20)	Group I normoalbuminuric (N=30)	Group II microalbuminuric (N=25)	Group III macroalbuminuric (N=14)
Microalbumin concentration (mg/L)				
Median	------	7.85	71.2	304.9
IQR		(6.10-12.95)	(46.30-100.05)	(187.80-426.18)
Creatinine in urine (mg/dL)				
Median	------	97	87	45.6
IQR		(69.00-132.00)	(60.00-109.25)	(34.60-57.95)
ACR (mg/g)				
Median	------	9.9	71.6	558
IQR		(7.50-14.88)	(43.50-134.35)	(515.73-840.73)
u-L-FABP (ng/L)				
Median	4.95	6.5	160.2	1350
IQR	(2.9-32.08)	(2.98-51.50)	(71.25-245.95)	(777.30-1455)
u-L-FABP (ng/g creatinine)				
Median	------	6.05	168.78	2301.76
IQR	------	3.22-49.59	98.66-305.76	1994.2-2737
u-NAG (u/L)				
Median	21.55	23.40	26.80	41
IQR	(7.05-37.6)	(11.35-53.6)	(16.05-51.35)	(30.25-85)
u-NAG (u/g creatinine)				
Median	------	24.65	36.89	87.7
IQR		(12.75-60.92)	(19.91-56.7)	40.04-167.02)

Table (3): Comparison between patient groups regarding duration of diabetes

Duration (years)	Group I normoalbuminuric (N=30)	Group II microalbuminuric (N=25)	Group III macroalbuminuric (N=14)	F	p-value
Range	**1-15**	**7-20**	**14-30**	**49.073**	**<0.001****
Mean ± SD	**7.60±3.94**	**12.00±3.49[a]**	**19.64±3.86[ab]**		

F: ANOVA test.
**p-value < 0.001 HS (highly significant).
Post-hoc test: (LSD)
a: Significant difference between group I (p-value <0.05).
b: Significant difference between group II (p-value <0.05).

Table (4): Comparison between studied groups regarding glycemic parameters.

	Control Group (N=20)	Group I normoalbuminuric (N=30)	Group II microalbuminuric (N=25)	Group III macroalbuminuric (N=14)	F	p-value
FPG (mg/dL)						
Range			126-368		34.883	<0.001**
Mean±SD	76.50±4.72	174.14±8.94[a]	274.28±6.14[ab]	199.50±2.88[ac]		
HbA_1c%						
Range	4.5-5.6	6.9-10.5	7.9-15.1	8.1-12.2	52.923	<0.001**
Mean±SD	5.22±0.38	8.78±1.91[a]	10.06±1.24[ab]	9.14±0.71[ac]		
Urea (mg/dL)						
Range	15-27	18-41	20-62	22-75	13.72	<0.001**
Mean ± SD	24.50±3.35	26.32±4.86	44.40±8.73[ab]	56.21±10.19[abc]		
Creatinine (mg/dL)					16.45	<0.001**
Mean ± SD	0.75±0.09	0.78±0.11	1.56±0.24[ab]	2.14±0.4[abc]		
e-GFR ($ml/min/1.73m^2$)					21.71	<0.001**
Range	75-114	74-106	55-79.83	35-64		
Mean±SD	97.33±8.1	94.5±6.3	68.75±5.2[ab]	55.28±2.65[abc]		
TG (mg/dL)					3.581	0.017*
Mean ± SD	105.05±9.94	138.10±2.52[a]	163.57± 6.85[ab]	222.68±4.19[abc]		
TC (mg/dL)					6.404	<0.001**
Range	120-193	92-273	116-299	131-334		
Mean ± SD	165.05±19.92	203.90±45.44[a]	211.04±44.42[a]	215.50±47.66[a]		
HDL-C (mg/dL)					2.977	0.036*
Range	36-55	30-57	30.7-53.5	19-59		
Mean ± SD	43.68±5.07	42.96±9.12	40.39±7.80	37.40±8.85[abc]		
LDL-C (mg/dL)					6.316	<0.001**
Mean ± SD	101.40±12.75	101.61±3.64	129.32±4.20[ab]	135.51±5.99[ab]		

Post-hoc test: (LSD):
a: Significant difference between control group (p-value <0.05).
b: Significant difference between group I (p-value <0.05).
c: Significant difference between group II (p-value <0.05).

Table (5): Correlation analysis between u-L-FABP (ng/L) & u-NAG (u/L) and other studied parameters in all diabetic patients as a whole.

Parameters	u-L-FABP (ng/L)		u-NAG(u/L)	
	rs	p-value	rs	p-value
u-L-FABP (ng/L)			0.518	<0.001**
u-NAG (u/L)	0.518	<0.001**		
Age (years)	0.135	0.269	0.194	0.110
Duration (years)	0.258	0.032*	0.691	<0.001**
FPG (mg/dL)	0.217	0.074	0.023	0.850
HbA_1c%	0.034	0.782	0.012	0.919
Urea (mg/dL)	0.329	0.006**	0.220	0.069
Creatinine (mg/dL)	0.639	<0.001**	0.223	0.065
e-GFR (ml/min/1.73m^2)	-0.390	0.001**	-0.113	0.354
ALT (u/L)	0.150	0.219	0.140	0.252
AST (u/L)	0.048	0.695	0.005	0.965
Total cholesterol (mg/dL)	0.205	0.125	0.105	0.388
TG (mg/dL)	0.011	0.931	0.168	0.167
HDL-C (mg/dL)	-0.026	0.829	-0.079	0.521
LDL-C (mg/dL)	0.210	0.119	0.182	0.133
Microalbumin concentration (mg/L)	0.173	0.207	0.027	0.845
ACR	0.217	0.111	0.102	0.461

Table (6): Correlation between u-NAG (u/g creatinine) and u-L-FABP (ng/g creatinine), HbA_1c% and ACR in all studied patient groups.

Studied Parameters	u-NAG to (u/g creatinine)							
	Group I		Group II		Group III		All patients	
	rs	P-value	rs	P-value	rs	P-value	rs	P-value
u-L-FABP (ng/g creatinine)	0.424	0.020*	0.663	<0.001**	0.820	<0.001**	0.627	<0.001**
HbA_1c %	0.094	0.622	0.185	0.375	0.329	0.251	0.035	0.776
ACR	0.140	0.460	0.044	0.835	0.191	0.513	0.379	0.002**

Table (7): Correlation between u-L-FABP (ng/g creatinine) and u-NAG (u/g creatinine), HA_1c % and ACR in all studied patient groups.

Studied Parameters	u-L-FABP (ng/g creatinine)							
	Group I		Group II		Group III		All patients	
	rs	P-value	rs	P-value	rs	P-value	rs	P-value
u-NAG (u/g creatinine)	0.424	0.020*	0.663	<0.001**	0.820	<0.001**	0.627	<0.001**
HbA_1c %	0.047	0.803	0.230	0.269	0.461	0.097	0.130	0.286
ACR	0.193	0.306	0.153	0.465	0.222	0.446	0.800	<0.001**

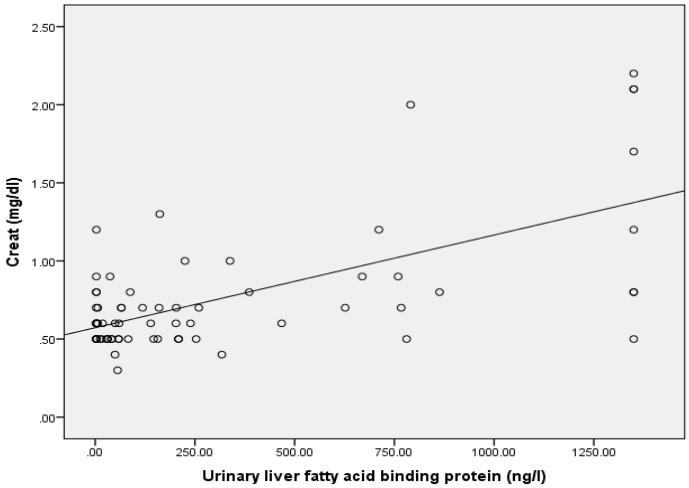

Fig. (1): Scatter plot between u-L-FABP (ng/L) and serum creatinine in all patients group.

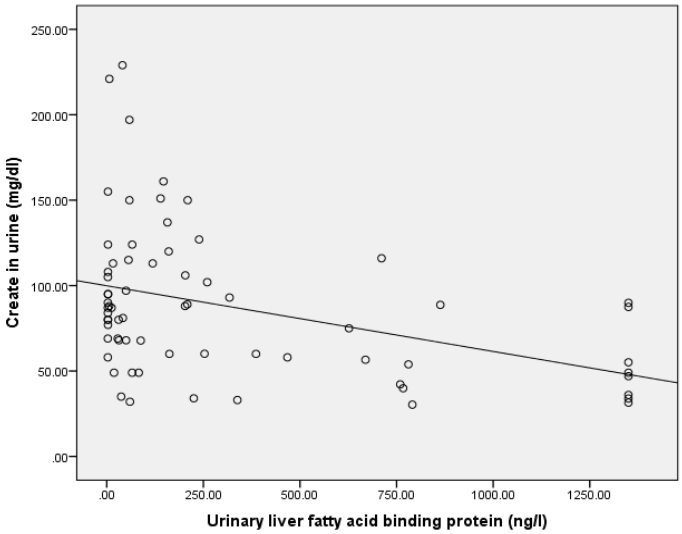

Fig. (2): Scatter plot between u-L-FABP (ng/L) and e-GFR in all patients group.

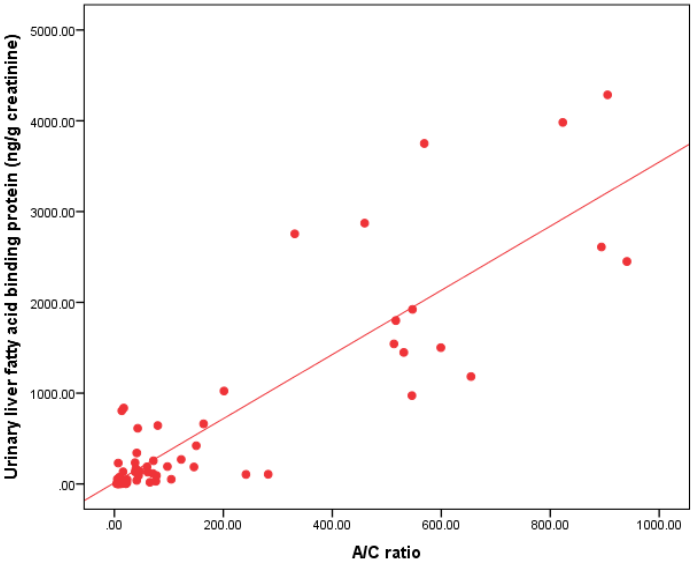

Fig. (3): Scatter plot between u-L-FABP (ng/g creatinine) and ACR in all patients group.

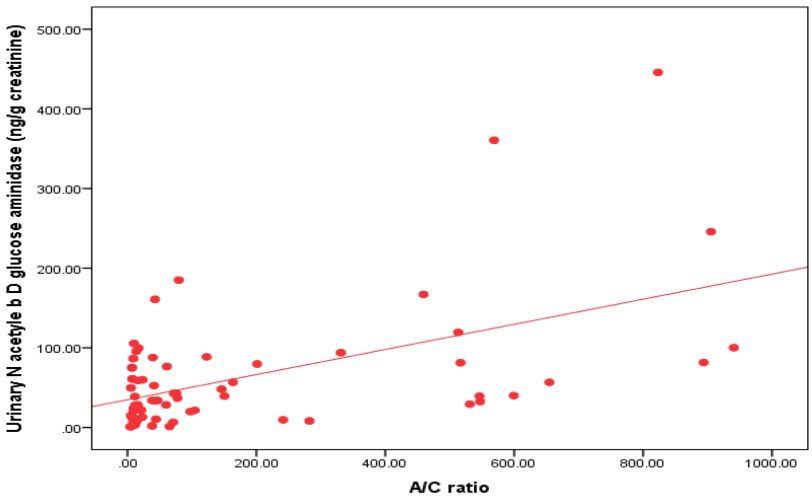

Fig. (4): Scatter plot between u-NAG (u/g creatinine) and ACR in all patients group.

DISCUSSION

Statistical analysis in our study revealed a significant decline in kidney functions as indicated by elevated serum creatinine and urea concentration and reduced e-GFR in diabetic subgroups II and III (microalbuminuric and macroalbuminuric, respectively) as compared to healthy control group and normoalbuminuric group I ($p<0.05$). These alterations in kidney function tests were more significant in macroalbuminuric group with the highest duration of diabetes, in comparison to microalbuminuric group ($p<0.05$). These results indicate that in early deterioration of kidney function coincided with the development of microalbuminuria and become exacerbated with progression to macroalbuminuria.

Our findings were not in agreement with the study of [16, 17] who found significant alteration of kidney function tests in their studied type 2 diabetic patients with normoalbuminuria as was reported before by [18]. In addition, in the study of [19], while e-GFR showed significant reduction in microalbuminuric group, serum creatinine significantly elevated only, with the development of macroalbuminuria.

However, our results were in accordance with the findings of [20], who detected such impairment of renal function among type 2 diabetic patients with microalbuminuria. These findings support the explanation of [21] that once microalbuminuria is present, creatinine clearance decreases due to failing of GFR with increasing levels of urea and creatinine.

The interest for the use of potentially sensitive biomarkers for early detection of DN derives from the observation that patients with type 2 diabetes pass through a period of pre-diabetes and so, may experience renal impairment at the time of diagnosis [18]. Urinary enzymes have been used as valuable clinical tools to assess the preclinical stage of DN, monitor disease progression and detect early impaired renal function in diabetic patients [19].N-acetyl-β-D-glucosaminidase (NAG) is the most widely used urinary enzyme for assessment of many renal diseases [22].

Previous studies have reported that compared to controls, increases in u-NAG excretion already occurs in patients with type 2 DM with normal to mildly increased albuminuria, reflecting early tubular dysfunction [23, 24].

In our study, we were surprised as we did not find statistically significant differences in u-NAG excretion between enrolled diabetic subgroups and controls as well as between various diabetic groups with increasing categories of albuminuria, although increases in urinary enzyme levels were detected in association with progression of albuminuria, however, of non-significance ($p=0.062$). There were also non-significant difference in urinary levels of the enzyme when all enrolled diabetic patients were compared with controls as a whole.

However, when comparing the median value of u-NAG expressed in relation to urine creatinine concentration (u/g), a highly significant increase was found only in diabetic group with macroalbuminuria in comparison to normoalbuminuric and microalbuminuric groups ($p<0.001$ and $= 0.003$, respectively). The proportional increases in the median value of u-NAG (u/g) between diabetic subgroups were found to be about 3.5- and 2-fold increases in macroalbuminuric group, compared to normo-and microalbuminuric groups, respectively.

Accordingly, our results were not matched with previous studies in type 2 diabetic patients that found in comparison to controls, significant increases in u-NAG excretion, reaching 9 fold in normoalbuminuric group [23] or among microalbuminuric patients [24], or rather, reaching 8- and 10- folds increases in normoalbuminuric patients with more than 10 and 15 years duration of diabetes, respectively; with more

significant increases by 16 and 18 folds in patients with micro-and macroalbuminuria, respectively [19].

Moreover, in contrary with our findings, [17, 25] found significant increases in u-NAG excretion in all diabetic subgroups in comparison to healthy controls with a characteristic increasing trend parallel to the development of albuminuria in type 2 diabetic patients, and suggested that tubular dysfunction as evidenced by an increase in u-NAG excretion, is already developed in earlier stages of DN and this becomes exacerbated with worsening the degree of albuminuria. Nevertheless, [19] did not find such significant increase in u-NAG activity in all normoalbuminuric patients in their study, except in only patients with long duration of diabetes, above 10 years.

As confined to the limited number of enrolled type 2 diabetic patients in our study, we suggested that u-NAG determination might be a potential prognostic marker, indicating the severity of renal involvement in diabetic kidney disease specially tubular dysfunction, rather than a predictor of disease development and progression.

The conducted studies demonstrated that u-NAG excretion positively associated with ACR as well as diabetes duration [19, 26], serum levels of creatinine [17] or e-GFR (negative correlation) [26], percentages of HbA_1c [17, 26] and fasting blood glucose levels [26].

In our study, u-NAG excretion did not show significant correlations with all estimated parameters in different studied diabetic subgroups except with u-L-FABP {group I ($p=0.005$), group II ($P<0.001$) and group III ($P=0.005$)}. Significant positive correlations were also demonstrated with u-L-FABP and duration of diabetes when all patients are included as one group ($P<0.001$). U-NAG excretion also showed a significant direct correlation with ACR when its levels are expressed in relation to urine creatinine in the same patient group including all enrolled diabetic cases ($p=0.002$). However, u-NAG did not show significant associations with other evaluated parameters of renal dysfunction and glycemic control.

In alignment with our results, [19, 26] found that u-NAG excretion was strongly associated with the duration of type 2 diabetes and ACR. Also, [27] found significant positive correlation between u-NAG (u/g) and ACR in their studies on type 2 diabetes. These results reemphasized the importance of sustained increase in UAE in the pathogenesis as well as the diagnosis of diabetic kidney disease and also, the highly association of total u-NAG activity with the level of proteinuria in both glomerular and tubulointerstitial pathologies [28].

From our results, we can suggest that u-NAG excretion is most probably reflect the severity of renal involvement, depending on the presence of sustained excessive increase in UAE that is strongly associated with both glomerular and tubulointerstitial pathologies of diabetic kidney disease.

Moreover, u-NAG was found to have no significant discriminative abilities to differentiate between diabetic subgroups and controls, or even to discriminate between diabetic groups with increasing levels of albuminuria, at best calculated cutoff values, assuming the significance at <0.05. These findings indicated that determination of u-NAG excretion as a biomarker of tubular damage secondary to diabetes of no diagnostic value efficiency to discriminate diabetic patients from healthy control subjects, or to distinguish between diabetic subgroups with various degrees of renal involvement and varying levels of albuminuria.

Our results were not in agreement with [11] who assess the diagnostic performance of u-NAG excretion in type 2 diabetic patients with increasing levels of albuminuria and found that at a cutoff value of 3 u/L, u-NAG was significant in discriminating diabetic groups with microalbuminuria and macroalbuminuria from control group, but did not possess the capability to differentiate those with normoalbuminuria from healthy controls. This cutoff value has demonstrated a specificity of 96.1% and (efficacy) of 99.9%, as well as a specificity of 100 % and accuracy of 100%, respectively.

However, these results demonstrated that u-NAG although is known to be an early marker of tubulointerstitial damage, it did not show the diagnostic efficacy to identify diabetic patients at clinical quiescence stage of diabetic kidney disease.

As was expected, the correlation analysis in our study revealed highly significant positive associations between u-NAG excretion and the urinary excretion of the other tubular damage marker originating also from the proximal renal tubules, liver-fatty acid binding protein, in all studied diabetic patients as a whole and in different diabetic subgroups with increasing levels of albuminuria. This strong relationship may support the clinical utility of such tubular damage markers in evaluating renal involvement in diabetes.

In the current study, there were highly significant increases in the urinary levels of L-FABP (u-L-FABP), reaching a 18-fold in our studied type 2 diabetic patients as a whole, as compared to control group ($p<0.001$) and also, in microalbuminuric and macroalbuminuric diabetic subgroups as compared to both control and normoalbuminuric groups ($p<0.001$). The highest increase in u-L-FABP excretion was associated with the more advanced stage of disease progression, macroalbuminuria ($p<0.001$).

In addition, our results demonstrated that, in comparison to healthy controls, u-L-FABP excretion increased 32-fold with the development of microalbuminuria and 270 fold with progression to macroalbuminuria. Moreover, in comparison to normoalbuminuric group, u-L-FABP (ng/L and ng/g) was increased about 25 and 28 folds, respectively in diabetic group with microalbuminuria as well as 208

and 308 folds, respectively in macroalbuminuric group.

These results emphasized that elevated urinary excretion of L-FABP is a significant clinical marker for early detection of tubulointerstitial damage induced by structural and functional changes secondary to diabetes and progressive increases in u-L-FABP are associated with the severity of the disease that leads to increased synthesis and levels of L-FABP inside the cells to bind lipid peroxides overloading the cells as a result of oxidative stress and transfer them to urinary spaces to be excreted into urine through the damaged proximal tubule cell membranes.

Although our results were in agreement to a large extent with several clinical studies conducted on type 2 diabetic patients, these studies in contrary, have demonstrated the elevation of u-L-FABP at an earlier stage of DN (normoalbuminuria), with significantly higher urinary levels with progression of renal involvement {[26] ($p<0.05$), [29] ($p<0.001$) and [20] ($p=0.001$)}.

The investigators concluded that u-L-FABP is a suitable predictor for the development and progression of DN. In addition, it was supposed the metabolic status in type 2 diabetic patients is one of the important reasons why the L-FABP excretory levels are increased early in patients with normoalbuminuria [29].

However, in the study of [29], elevated levels of u-L-FABP were detected in only 8% of normoalbuminuric patients with normal kidney function and so, they considered u-L-FABP as a potential marker for predicting the prognosis of kidney function in diabetic kidney disease.

In our study, increases in u-L-FABP level was associated with renal insufficiency and decreased functional capacity of the kidney in diabetic patients showing microalbuminuria as indicated by elevated serum urea and creatinine and reduced e-GFR.

It was reported that in presence of albuminuria, excess cytosolic FFAs lead to tubulointerstitial damage and cell dysfunction and even cell death by promoting endoplasmic reticulum stress and excess production of ROS, processes collectively designated as"lipotoxicity" [30].This may explain why high significant levels of u-L-FABP were detected in our study from the microalbuminuric stage of DN. Therefore, we suggested that the use of u-L-FABP alone, or together with microalbumin, is beneficial for early diagnosis and monitoring the progression of diabetic kidney disease.

Correlation analysis in the present study did not revealed significant associations between elevated levels of u-L-FABP and altered glycemic parameters (FBG and HbA_1c %) representing risk factors for the development of DN, as that detected by [29] and [20] and lead to the suggestion that a good glycemic control may offer a potential mechanism that protects the kidney function in type 2 diabetes [29].

Similarly, no significant correlations were found between u- L-FABP and parameters of dyslipidemia observed among diabetic patients in our study. We suppose the inclusion of other factors influencing plasma levels of such metabolic parameters, in addition to the renal pathology involved.

Furthermore, when ROC analysis was employed to evaluate the diagnostic performance of u-L-FABP, unlike u-NAG, u-L-FABP showed a significant discriminative power to distinguish between either microalbuminuric or macroalbuminuric diabetic patients and healthy controls ($p<0.05$) and also to differentiate between diabetic subgroups with increasing levels of albuminuria, reflecting different stages of nephropathy ($p<0.05$). However, u-L-FABP did not show a diagnostic efficiency to distinguish diabetic patients with normoalbuminuria from healthy controls.

The diagnostic performance of u-L-FABP in discriminating between either microalbuminuric or macroalbuminuric patient groups and controls was best shown at a cutoff value of >37.2 and >92.2 ng/L with a diagnostic specificity of 90% and 100% accuracy (efficacy) of 96% and 100%, respectively. In addition, u-L-FABP at cutoff values of >28.5 and >386.1 ng/L, demonstrated a diagnostic sensitivity of 100%, and accuracy of 87.9% and 99.7% in predicting the prognosis of microalbuminuria and macroalbuminuria, respectively.

These findings demonstrated that determination of u-L-FABP has the diagnostic efficacy to identify (diagnose) diabetic patients with incipient DN and its levels can be used as efficient measure for prediction of disease progression.

CONCLUSIONS

Our results reemphasized the clinical usefulness of u-L-FABP to screen for tubulointerstitial damage as a consequence of diabetes. In addition, our results suggested that determination of u-L-FABP excretion might be useful as a non-invasive relevant test for early detection of DN and altered functional capacity of the kidney and prediction of disease progression. Furthermore, the use of u-L-FABP as a specific proximal tubular damage biomarker alone, or together with microalbumin, is beneficial for early diagnosis and monitoring of DN, compared to u- NAG excretion.

RECOMMENDATIONS

We recommended the performance of extensive clinical studies on large number of diabetic patients and with other established sensitive tubular damage markers, taking into consideration a more specified criteria for both investigated patients and estimated

urinary tubular biomarkers to verify their reliability as early indicators of DN.

In addition, follow-up studies are also needed for newly diagnosed cases with diabetes to demonstrate significant changes in the pattern of urinary excretion of these biomarkers along the disease course and to identify emerged factors that may influence their urinary levels. This may be helpful to know more about the pathophysiology and associations of these biomarkers and to re-set diagnostic cutoff values that predict the development and progression of renal dysfunction irrespective of UAE and also, to assess responses to different therapies.

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