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U-LFABP AS A PREDICTIVE MARKER FOR PROGNOSIS OF DIABETIC KIDNEY DISEASE IN EGYPTIAN PATIENTS

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1. ABSTRACT

This study was aimed to evaluate the level of urinary liver-type fatty acid binding protein (u-LFABP) as a marker of diabetic kidney disease and to study its correlation with other kidney parameters in Egyptian patients with chronic type 2 diabetes mellitus (T2DM). A total of 70 T2DM subjects were divided into three groups: diabetics with normal kidney function and normal blood pressure (DM; 20 patients), diabetics with microalbuminureia and high blood pressure (early-DN; early diabetic nephropathy) (20patients) and diabetic kidney disease (DKD) with microalbuminuria and high blood pressure (30 patients), with 20 non-diabetic control subjects. U-LFABP levels were measured by ELISA technique. Results showed that u-LFABP level was highly significant increased (P<0.001), this increase was more pronounced in early DN group and in DKD patients compared to normal control indicating tubular damage. The levels of u-LFABP increased gradually with declining renal function and reduced e-GFR. U-LFABP showed a positive correlation with s- creatinine, UAER and IL-6 (r 0.55, 0.32 and 0.56; respectively) and a significant negative correlation with e-GFR (r= 0.54, P<0.05) in the early DN patients' group. In DkD patients' group a significant positive correlation was recorded between u-LFABP and fasting plasma glucose, HbA1c, fasting insulin, s- creatinine, UAER and IL-6 (r= 0.35, 0.57. 0.45, 0. 67, 0.70 and 0.77; respectively) and negative correlation with e-GFR (r= 0.66, P<0.001). As a result of receiver operating characteristic (ROC) analysis, u-LFABP appeared to be a more sensitive marker for the detection of early-DN and also for the prediction of DKD progression in diabetic patients.

KEYWORDS: Diabetic nephropathy, u-LFABP, diabetic kidney disease.

1. INTRODUCTION

The incidence and prevalence of diabetes mellitus (DM) have grown significantly throughout the world, primarily type 2 DM (T2DM). This increase in T2DM disproportionately affects less developed countries, which also have fewer resources to deal with such patients. The increase in the number of people developing diabetes will also have a major impact on dialysis and transplant needs.[1]

Recent estimates suggest that 1 in 12 of the global population suffers from diabetes mellitus. Approximately 40% of those affected will go on to develop diabetes-related chronic kidney disease (CKD) or diabetic nephropathy (DN).[2] Progression of chronic kidney disease is inevitable. However, the last decade has witnessed tremendous achievements in this field, the dream of withholding this progression is about to be realistic.[3]

Traditional risk factors such as age, gender, body mass index, smoking status, dyslipidemia and CKD have been shown to be associated with the incidence of hypertension in persons with and without diabetes. The kidney plays a significant role in the regulation of blood pressure (BP) by controlling blood volume and extracellular electrolytes, glomerular hemodynamics and the renin-angiotensin system.[4]

The routine classical evaluation of diabetic nephropathy includes the appearance of microalbuminuria, decreased creatinine clearance and increased serum creatinine. Although microalbuminuria has been considered as the gold standard for early diagnosis of DN in clinical practice, 29.1–61.6 % of individuals with T2DM could have renal impairment even before the onset of microalbuminuria.[5] However, serum creatinine also depends on creatinine production, external elimination and tubular handling. Moreover, tubular involvement may precede glomerular involvement because several tubular proteins and enzymes are detectable even before the appearance of microalbuminuria and a rise in serum creatinine.[6-7]

Several glomerular and tubular biomarkers could be used as markers for earlier, specific and accurate prediction or progression of nephropathy in patients with diabetes have been identified and they are becoming increasingly important in clinical diagnostics.[8–9]

Urinary liver-type fatty acid–binding protein (u-LFABP) is an intracellular carrier protein, 14 kD,expressed in the proximal tubules of the human kidney and liver. It participates in fatty acid metabolism.[10] It is a promising indicator of tubular function, but not glomerular damage.[11] Its urinary excretion is associated with structural and functional tubular damage.[12] Thereby offers to be a potential clinical marker that can identify patients who are likely to experience deterioration of renal function in the future.[13]

DN has not been traditionally considered an inflammatory disease, however, studies have shown that kidney inflammation is crucial in promoting the development and progression of DN. Inflammation may be a key factor which is activated by the metabolic, biochemical and haemodynamic derangements known to exist in the diabetic kidney.[14]

Neuropeptide Y (NPY) is induced in peripheral tissues and can modulate inflammation by regulation of the function of adipose tissue macrophages.[15] NPY causes a decrease in glomerular filtration rate, aldosterone concentration, and plasma renin activity by stimulating NPY receptors in the kidney.[16]

In order to delay the onset of chronic kidney disease, systematic screening and appropriate management are needed to evaluate the progression of renal damage in diabetic patients. In accordance, this study was constructed to evaluate the clinical usefulness of u-LFABP as a prognostic biomarker in impaired diabetic nephropathy with microalbuminuria in type 2 diabetic Egyptian patients. As well as correlating it with some renal and inflammatory markers in diabetic nephropathy patients.

2. SUBJECTS AND METHODS

2.1. Subjects

A total of 90 subjects; 20 normal control subjects and 70 patients with type 2 diabetes mellitus from Ghamra Hospital were enrolled in this study. Written informed consent was obtained from all patients after full explanation of the procedure used. The diagnosis of type 2 diabetes mellitus was performed according to the World Health Organization (WHO) criteria. Diabetic nephropathy (DN) was diagnosed by measuring the estimated glomerular filtration rate (e-GFR< 60 ml/min/1.73 m^2) and the urinary albumin excretion rate (UAER) at baseline. In this study, patients with microalbuminuria were classified as having UAER 30–300 mg/24 hr at collection time.[17] The diabetic patients were subdivided into three groups: diabetics with normal kidney function and normal blood pressure (DM; 20 patients), diabetics with microalbuminuria and normal blood pressure (Early-DN; 20patients) and diabetic kidney disease with microalbuminuria and high blood pressure (DKD;30 patients). The participants in the diabetic groups were uncontrolled type 2 diabetic Patients, they were treated with different drugs as follows: Anti-hypertensives (Epilat retard, Capotein, Natrilix), hypoglycemic agents (Diamicron, Daonil, Insulin Mixtard), anticoagulants (Heparin, Dindivan) and Aspirin. Dose adjusted according to the state of each patient. None of control subjects were taking any medications known to affect glucose metabolism. The duration of the disease was 6.1±0.27 years in normoalbuminuria (DM), 10.1±0.75 years in microalbuminuria patients (Early-DN) and 15± 0.61 years in DKD patients.

Exclusion criteria of the subjects were as follows: diabetic macrovascular complications, thyroid dysfunction, or taking drugs which affect glucose and lipid metabolism, chronic hepatitis, recent inflammatory disease, acute trauma, pregnancy, malignancy and history of drug abuse. Before starting, informed consent was obtained from all participants. Approval was taken from Ain Shams University.

2.2 METHODS

Standardized questionnaire was used for history of present and past illness, medication, age, sex. Diabetes duration and blood pressure (BP) was performed on each subject. After obtaining written informed consent, fasting blood samples (approximately 5 ml) were collected and immediately centrifuged at 3000 rpm for 10 min. Sera were rapidly separated, aliquoted, for the measurements of levels of blood urea nitrogen (BUN), creatinine (Cr), total cholesterol (TC), triacylglycerol (TAG), high-density lipoprotein cholesterol (HDL-C), low-density lipoprotein cholesterol (LDL-C), interleukin-6 (IL-6) and neuropeptide Y(NPY).

Another part of blood was taken on EDTA for determination of HbA1c levels. For glucose estimation, potassium fluoride was added to tubes. Hemolysed samples were excluded. Fresh morning urine samples were obtained for measurement of albumin, Cr concentration, and u-LFABP. Urine was collected and centrifuged at 1500 rpm for 10 min to remove cells and supernatant was kept until analyzed. All samples were thawed once, each individual provided a 24-h urine sample and fasting blood sample at baseline. The serum and urine samples were kept at–08^{0}C if they were not analyzed immediately.

2.3. Biochemical investigations

Commercial kits were purchased from BioMed. (Egy Chem, Egypt). Plasma glucose concentrations were assayed at once by the glucose oxidase method according to Trinder.[18] Plasma insulin concentration was determined using a commercially available enzyme linked immunosorbent assay (ELISA) kit (Biosource

Europe SA, Nivelles, Belgium) based on the method of Flier *et al.*,[19] Insulin resistance was defined by homeostasis model assessment for insulin resistance (HOMA-IR). HOMA-IR was calculated according to Matthews *et al.*,[20] HbA1c % was measured according to the method of Grey *et al.*[21] using an immunoturbidimetric assay on Dimension RxL Max (Dade Behring).

Serum IL-6 and NPY were analyzed by ELISA using the commercially available ELISA kits (Quantikine, R&D Systems) and followed the manufacturer's recommendations.

The microalbumin assay (turbidimetric immunoassay) was used for the quantitative measurement of albumin in the urine on the Architect c8000TM System. The kit was provided by Abbott Diagnostics based on the method of Justesen *et al.*,[22] The renal tubular injury marker u-LFABP (human u-LFABP ELISA Kit, Hycult Biotech Inc., NL, US), was measured in 24 h urine sample.[13]

Lipid parameters, including TC, TAG, HDL-C, LDL-C and kidney functions, including BUN, Cr, were detected by biochemical autoanalyzer. Estimated Glomerular filtration rate (e-GFR) was calculated using the Cockcroft and Gault formula[23] and was normalized per 1.73 m^2 of body surface area.

Hypertension was defined as a systolic BP of ≥140 mmHg and/or a diastolic BP of ≥90 mmHg and/or a history of hypertension with current use of antihypertensive medications.[24]

2.4. Statistical analysis

Statistical analysis was performed using the Statistical Package for the Social Science (SPSS) for Windows (version 22.0, Chicago, IL, USA). Data are presented as means ±SD. The data were analyzed by one-way analysis of variance (ANOVA). A *P* value less than 0.05 was considered statistically significant. Pearson's correlation coefficient analysis was used to determine the correlations between serum u-LFABP and the different diabetic and kidney function studied parameters. Also receiver operating characteristic (ROC) curve was performed to define the sensitivity and specificity of u-LFABP as a predictive biomarker.

3. RESULTS

The results of the current study were summarized in (Table 1). It showed that, fasting plasma glucose, plasma insulin, HOMA-IR and HbA1c% showed a significant increase (P<0.05) in type 2 diabetic patients, early DN and DKD compared to normal control subjects.

Renal biomarkers in table 1 demonstrate that BUN, s-Cr, u-Cr and e-GFR rate were not significantly changed in DM compared to control subjects. On the other hand, BUN and s-Cr were significantly increased (P<0.05) in early DN and highly significant increased (P<0.001) in DKD. e-GFR and u-Cr were significantly decreased (P<0.05) in early DN and DKD patients compared to control group.Urinary albumin excretion rate (UAER) showed a non- significant change in diabetic patients. While, in early DN and DKD patients, it was dramatically increased compared to control subjects.

Data in table 1 also showed that, DM group has a significant increase in TC compared to control group (P< 0.05). While in early DN and DKD groups a significant increase (P< 0.05) was observed in TC, TAG and VLDL-c with a highly significant increase in LDL-c (P<0.001) and a significant decrease in HDL-c compared to control subjects.

Figure 1 illustrates the percent change of u-LFABP, NPY and IL-6 in the studied groups.u-LFABP and NPY showed a non-significant change with a highly significant increase in IL-6 in DM patients group (19,18 and 139%, respectively).

While in early DN patients, u-FABP, NPY and IL-6 were Markedly increased (70, 76 and 292%, respectively)(P<0.05). This increment was more pronounced in DKD patients group (172, 141 and 403%, respectively)(P<0.001).

Correlation between parameters

Table (2) showed the Pearson's correlation coefficients (r) between u-LFABP level and some biochemical parameters in the patient groups. There is a significant positive correlation between u-LFABP and s-Cr, UAER and IL-6 (r 0.55, 0.32 and 0.56; respectively) and a significant negative correlation with e-GFR (r= 0.54, P<0.05) in the early DN patients' group. On the other hand, In DKD patients' group a significant positive correlation was recorded between u-LFABP and FBG, HbA1c, fasting insulin, s-Cr, UAER and IL-6 (r= 0.35, 0.57, 0.45, 0.67, 0.70 and 0.77; respectively) and negative correlation with e-GFR (r= 0.66, P<0.001). No correlations were observed in DM group, except for a slight increase in s-Cr and IL-6.

ROC Analysis of some biomarkers

ROC analyses were performed (Fig 2) and fig (3), it showed that, area under the curve (AUC) of u-LFABP for early DN was 0.56 with a cut-off value of 118.5 Pg/ml Vs 0.66 and 0.57 with a cut-off values 126.6ml/L and 872.5 Pg/ml for UAER and NPY respectively, while in DKD AUC of u-LFABP was 1.0 with a cut-off value 199.5 Pg/ml Vs 0.92 and 0.99 with a cut-off values 277.5 ml/L and 1999.0 for UAER and NPY respectively.

Table (1): Anthropometric data and some biochemical parameters for patients included in the study

Groups Parameters	Control	DM	Early-DN (Microalbuminuria+ normotensive)	DKD (Microalbuminuria+ hypertensive)
Number	20	20	20	30
Age(year)	48.0 ±5.1	55.0 ±5.2	53.0 ±6.0	55.0 ±6.0
Sex(male/femal)	M 11/ F 9	M 7/ F 13	M 9/ F 11	M 11/ F 19
Duration (year)	----------	6.1± 0.3	10.1± 0.8	15.0 ± 0.6
SBP(mmHg)	121.9 ± 12.3	124.2 ± 15.6	129.8 ± 22.4	140.0 ±11.2
DBP(mmHg)	74.3 ± 8.9	74.5 ± 11.4	74.9 ± 12.4	95.0 ±5.3
FBS(mmol/L)	4.9 ±0.54	14.1±3.4*	16.0 ± 0.1*	16.6± 0.2*
Plasma insulin (mU/L)	7.6±2.6	10.2± 2.9*	11.9± 1.6*	12.1 ±4.6*
HOMA-IR	1.5±3.1	5.9±0.25*	8.7 ± 0.4*	9.1±3.0*
HbA1C (%)	4.9± 0.04	8.8±1.4*	10.1 ±1.0*	10.6 ± 1.0*
BUN(mg/dl)	25.6± 5.6	28.2 ±7.1	48.7 ±6.0*	104.8 ±34.5**
s-Cr (mg/dl)	0.7± 0.2	0.7 ±0.2	1.2±0.2*	2.1 ±0.4**
u-Cr(g/l)	122.5±20.4	113.4±24.3	87.5±12.7*	71.8±13.6**
TC(mg/dl)	144.7±22.1	207.0 ±42.2*	219.0 ±33.4*	263.0 ±61.1*
TAG (mg/dl)	109.0 ±21.3	139.6±63.5	171.9±43.8*	178.4±44.6*
HDL-c (mg/dl)	49.3 ±10.4	37.8 ±5.3	33.7 ±7.2*	29.7 ±6.8*
LDL-C (mg/dl)	78.1±14.6	135.3±29.3	140.4±20.4**	165.7±34.6**
VLDL-C (mg/dl)	24.4 ±5.1	29.8 ± 6.4	40.8 ± 12.4*	59.7 ± 18.6**
UAER(mg/L)	10.7±2.7	29.2 ±7.4	256.8 ±43.9**	293.2± 40.0**
e-GFR(ml/min/1.73m^2)	106.7 ±15.8	96.9 ±17.3	65.4 ±12.9*	53.1 ± 10.9*
IL-6(g/ml)	0.8 ±0.1	1.8± 0.4*	2.7± 0.6**	3.7± 0.6**
NPY(pg/ml)	599.2±30.6	708.5±62.8	1055.1±62.8**	1434.6±164.4**
u-LFABP(pg/ml)	82.9 ± 11.9	98.2 ± 16.8	140.8 ± 15.9*	225.5± 28.1**

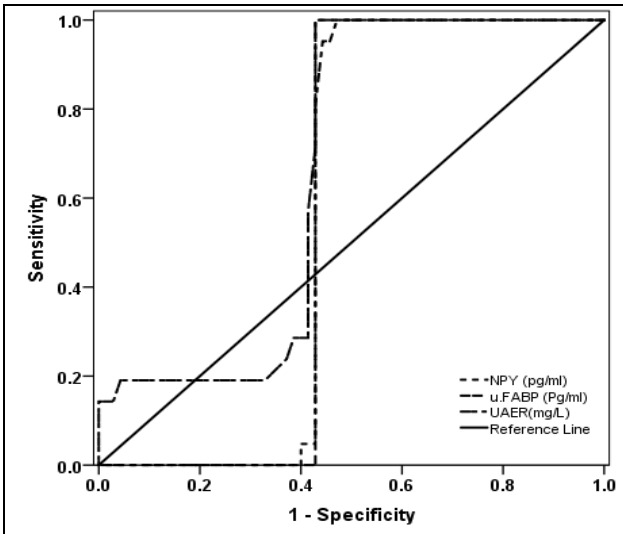
Data are expressed as mean ± SD.

SBP, systolic blood pressure; DBP,diastolic blood pressure;FBS, fasting blood sugar; HOMA-IR, homeostasis model assessment for insulin resistance;HbA1c, hemoglobin A_{1c}; BUN, blood urea nitrogen; Cr, creatinine; TC, total cholesterol; TAG, Triacylglycerol;HDL-C, high density lipoprotein -cholesterol; LDL-C, Low density lipoprotein cholesterol;VLDL-C, very low density lipoprotein-cholesterol; UAER, urinary albumin excretion rate; e-GFR, estimated glomerular filtration rate; IL-6, interleukin-6; NPY, neuropeptide Y; u-LFABP, urinary-liver fatty acid binding protein.*: Significant from control ($p<0.05$), **: High significant from control ($p<0.001$).

Table (2) Pearson's correlation coefficients (r) between u-LFABP level and some serum biochemical parameters in the patient groups

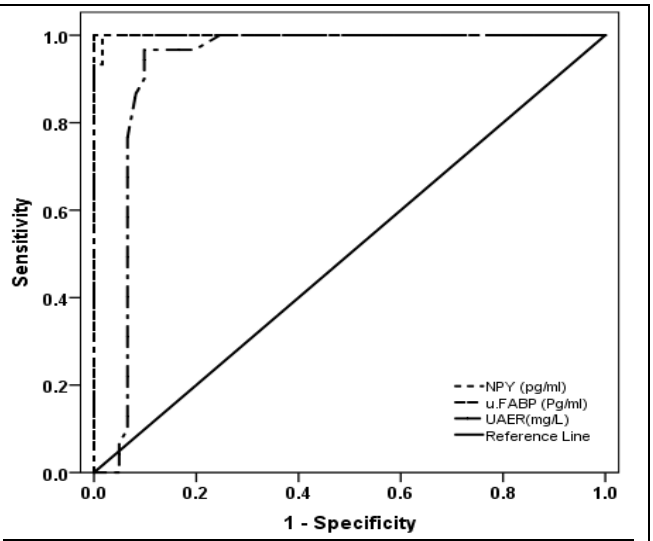
Groups Parameters	DM		Early-DN		DKD	
	r	P value	r	P value	r	P value
FBG	0.29	NS	0.32	NS	0.35	<0.05
HbA_{1c}	0.03	NS	0.21	NS	0.57	<0.001
Fasting-insulin	0.27	NS	0.41	Ns	0.45	<0.05
s- Cr	0.54	<0.05	0.55	<0.05	0.67	<0.001
UAER	----	----	0.32	NS	0.70	<0.001
eGFR	----	----	-0.54	<0.05	-0.66	<0.001
IL-6	0.51	<0.05	0.56	<0.05	0.77	<0.001

Pearson's correlation (r), NS: non- significant, P<0.001: highly significant



Variables	NPY (Pg/ml)	u-FABP (Pg/ml)	UAER (mg/L)
AUC	0.57	0.56	0.66
Cutoff	872.5	118.5	126.6

Figure (2): ROC curve for NPY, u-FABP and UAER in DN with microalbuminurea group.



Variables	NPY (Pg/ml)	u-FABP (Pg/ml)	UAER (mg/L)
AUC	0.99	1.0	0.92
Cutoff	1119.0	199.5	277.5

Figure (3): ROC curve for NPY,u-FABP and UAER in DKD group.

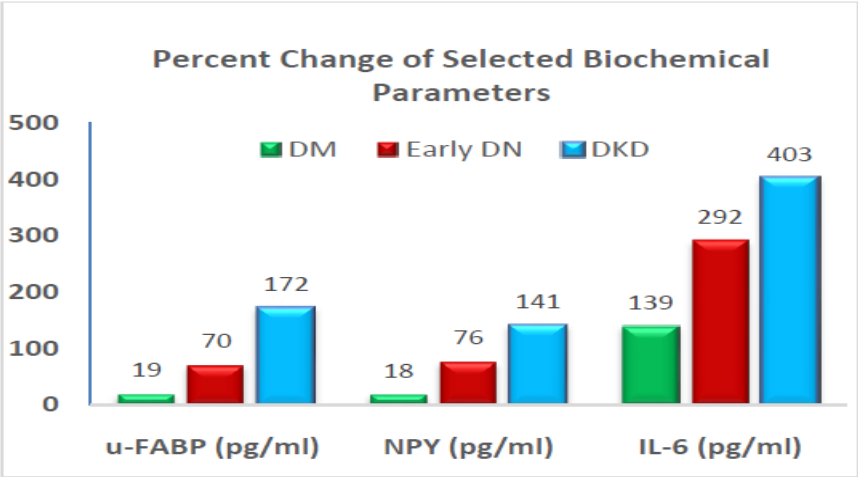


Figure (1): Percent change of u-FABP, NPY and IL-6 in DN, Early DN and DKD patient groups.

4- DISCUSSION

DN is a common and serious complication of diabetes associated with adverse outcomes of renal failure. Early and accurate identification of DN is of critical importance to improve patient outcomes.Changes in the renal tubules, which may be termed diabetic tubulopathy, are increasingly implicated in the development of progressive diabetic kidney disease. It has been reported that, in addition to the glomeruli, the renal tubules are heavily involved in the pathogenesis of DN.[25]

Renal dysfunction is reported to correlate with the degree of tubulointerstitial damage.[26] Although albuminuria per se reflects glomerular damage and subsequently induces renal tubulointerstitial damage, other factors and mechanisms, independent of albuminuria, must be involved in the development of tubulointerstitial damage under diabetic conditions.

To improve prognosis, it is important to predict the incidence of renal disease in type 2 diabetic patients before the progression of advanced nephropathy. Therefore, current study investigated the predictive value of u-LFABP, which is associated with renal tubulointerstitial damage, in renal prognosis.

In the present study, urinary u-LFABP was evaluated as a potential novel,valuable player associated with renal dysfunction in diabetic patients with microalbuminuria.The relation between u-LFABP and development of diabetic kidney disease could be explained in terms of the following: firstly, an increase in serum fatty acid (FA) could be another feature in diabetes. A lack of insulin function induces lipolysis and then releases the FA into circulation from white adipose tissue. The FA have been usually a source of energy for several types of cells, whereas an excess amount of FA is

likely problematic in our body. In fact, several studies indicate that serum FA could be involved in inflammation, endothelial dysfunction, and insulin resistance.[27]

Secondly, due to an increase in FA synthesis in the diabetic kidney. The lipid will deposit in the cells. These suggest that an increase in FA levels in serum and kidney could mediate the development of diabetic glomerular injury[28]. u-LFABP excretion is associated with structural and functional tubular damage. This was confirmed in a number of disease processes with significant proteinuria leading to tubular damage and chronic kidney disease (CKD) such as minimal-change nephrotic syndrome.[12]

The elevations in u-LFABP level parallel the degree of hyperglycemia, hyper-insulinemia and insulin resistance in DM, early-DN and DKD. Our findings are consistent with the emerging evidence that u-LFABP is engaged in diabetes and related complications, kidney dysfunction. In the present study, u-LFBP showed a nonsignificant increase in DM group, while a high significance increase in early diabetic nephropathy, this increase was synergized in DKD, suggesting that u-LFABP reflect tubulointerstitial damage therefore, predict the progression of deteriorating renal function. The current results were consistent with Temesgen and Zemenu[25] and Tsukasa *et al.*,[29] who clarified that in patients with type 2 diabetes mellitus, the elevation in u-LFABP levels were associated with the decrease of e-GFR.
Also, Nielsen *et al.*[30] reported that higher u-LFABP levels predicted allcause mortality in 165 patients with type 1 diabetes and normoalbuminuria, independent of urinary albumin excretion and other established risk factors. Additionally, Gianfranco and Yashpal[10] reported that, the increased filtered protein across the glomerular barrier imposes a tremendous stress on proximal tubules and causes accelerated excretion of u-LFABP from the tube compartment into the urine. As u-LFABP in the kidney has been postulated to represent an endogenous antioxidant capable of suppressing tubulointerstitial damage.

According to the aforementioned, u-LFABP accurately reflected the severity of diabetic nephropathy in type 2 diabetes, and its levels were high even in patients with normal trace amounts of excretion of albumin. This is supported by significant negative correlation between u-LFABP level and e-GFR in both early- DN and DKD patients' group. The previous results confirmed the role of u-LFABP in kidney dysfunction.

The results of the current study demonstrated a significant increasein TC, TAG, LDL-C and VLDL-Cwhile a significant decrease in HDL-C in early-DN and DKD patient Groups compared to control subjects.These results are in agreement with the previous studies which claimed that, dyslipidemia is a metabolic abnormality that is frequently associated with diabetes mellitus. Its prevalence is variable, depending on the type and severity of diabetes, glycaemic control, nutritional status, age and other factors.[31,32]

A common abnormality in type 2 diabetes is poor insulin secretion resulting in an increased lipolysis in adipocytes, increase in fatty acid transport to the liver which may cause an increase in VLDL-C.Also, insulin directly degrades the apo B, which is the major protein of VLDL particles and thus an insulin deficiency may increase the secretion of apo B and then VLDL.[31]

In the current study, u-LFABP was significantly increased. This increase was synergized with dyslipidemia, the level increased gradually with the progression of DN to reach its highest level in DKD patients ($p < 0.001$).

The results of the present study showed a significant increases in inflammatory biomarkers IL-6 and NPY in DM patients, which were augmented in early-DN patients and DKD ($P \leq 0.001$). This could be explained that patients with type 2 DM and overt nephropathy exhibit high levels of diverse acute phase markers of inflammation, including C-reactive protein (CRP) and IL-6.[33,34,35] Also, Insulin resistance may be a result of an overproduction of proinflammatory cytokines (eg, IL-6, tumor necrosis factor (TNF) and CRP) and a relative deficiency of anti-inflammatory cytokines.[36] In reality, diabetes and inflammation are found in the same individual more often than would occur by chance.
NPY is localized in the sympathetic nerves distributed in the juxaglomerular apparatus and in the renal tubular cells in the kidney.[26] It is therefore plausible that NPY is closely related to renal function, possibly acting as a potent vasoconstrictor, in patients with advanced diabetic nephropathy.[37] It causes decrease glomerular filtration rate, aldosterone concentration, and plasma renin activity by stimulating NPY receptors in the kidney.[38]

Results are in harmony with that of Asli *et al.* study to evaluated the relationship between the serum NPY levels and different stages of DN in patients with T2DM, they conclude that NPY and e-GFR were negatively correlated, so NPY may be an important predictor of advanced DN independent of the presence of microalbuminuria.[16]

In order to assess the predictive accuracy of u-LFABP for early detection of DKD, ROC analyses were performed. Accordingly, u-LFABP appeared to be a more sensitive marker for the detection of early stage of DN and also for the prediction the progression of DKD in patients with type 2 diabetes. These results in agreement with the results of Julia *et al.*[37] who calculated the sensitivity and specificity (81 and 83%, respectively) of u-LFABP in the prediction of renal failure in patients with DN.

5. CONCLUSION

u-LFABP considered as one of an additional tubular factors represents kidney state of diabetic patients. It could be used as a more sensitive markerin predicting progression of DKD than proteinuria in Egyptian Type 2 diabetes mellitus patients.

Conflict of interest

Authers have no conflict of interest.

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