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URINE NEUTROPHIL GELATINASE-ASSOCIATED LIPOCALIN AS AN EARLY BIOCHEMICAL MARKER OF MICROALBUMINURIA IN PREDICTING EARLY KIDNEY DAMAGE IN PATIENTS WITH TYPE 2 DIABETES MELLITUS

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Diabetic nephropathy is one of the microvascular complications of diabetes mellitus. The study was done to evaluate the diagnostic value of neutrophil gelatinase-associated lipocalin (NGAL) urine level as a biomarker for the early detection of nephropathy in type 2 diabetic patients. This study was performed on 150 patients with type 2 diabetes mellitus classified into three equal groups according to their urine albumin/creatinine ratio (ACR), including patients with normoalbuminuria (ACR < 30 mg/g creatinine), microalbuminuria (ACR = 30–300 mg/g creatinine) and macroalbuminuria (ACR > 300 mg/g creatinine). Fifty apparently healthy subjects were selected as a control group. Urine NGAL was estimated by a particle-enhanced turbidimetric immunoassay using Hitachi 917 analyzer. The serum level of glucose and creatinine was also estimated. The results showed that NGAL urine level was significantly elevated in diabetes patient groups with microalbuminuria and macroalbuminuria when compared to the control group and diabetes patient group with normoalbuminuria. The levels of urine NGAL correlated positively with microalbuminuria in patients with diabetes. Receiver operating characteristic curves revealed that urine NGAL had a higher diagnostic value for diabetic nephropathy early detection compared to ACR and microalbuminuria in patients with diabetes mellitus.

K e y w o r d s: gelatinase-associated lipocalin, diabetic nephropathy, albumin/creatinine ratio microalbuminuria.

Diabetes is a common endocrine disorder because of its worldwide increase in the prevalence rate. It subsequently increases incidence of macrovascular and microvascular complications including diabetic nephropathy (DN) [1].

Diabetes is found to be the major cause of end stage renal disease (ESRD) in patients who undergo dialysis [2, 3]. In a study from India, 46% of diabetic patients were found to have chronic kidney disease (CKD) [4].

DN is characterized by hypertension, albuminuria and decrease in glomerular filtration rate (GFR) leading to ESRD. DN is primarily a glomerular disorder but the decline in GFR is associated with severity of tubulointerstitial fibrosis and it is proposed to be a better predictor of DN than glomerular damage [5, 6].

The screening of DN is mainly based on assessment of microalbuminuria which is due to diabetes induced glomerular damage. The diagnostic accuracy of microalbuminuria in DN is being challenged by a large number of studies worldwide and it is being proposed that alternative biomarkers are also required for earlier identification of DN. The reason behind this is even diabetic patients with normoalbuminuria can develop significant degree of renal impairment [7, 8].

Recent studies have reported that the pathological changes in DN are associated with derangements in the glomerular and tubular compartments of the

kidney. Renal tubular and glomerular injury plays a major role in the development of DN and various biomarkers have been assessed for earlier detection of DN [9, 10].

Among them, neutrophil gelatinase-associated lipocalin (NGAL) seems to be a promising biomarker which is a 25 kDa protein with 178 aminoacids. It belongs to the lipocalin family of proteins and it is produced by renal tubules whenever there is structural injury to kidney [11].

In comparison to routine markers of renal function such as serum creatinine, cystatin C or urea, NGAL primarily reflects the structural damage of renal tubular cells. Also, serum creatinine which is done in most of the hospitals and considered to have a major diagnostic value in CKD cannot be taken solely as a marker since it is influenced by many other factors like age, muscle mass, race etc [12].

NGAL was found to be effective in early diagnosis of Acute Kidney Injury (AKI) following nephrotoxic insults like sepsis, cardiac surgery and following administration of contrast agents [13, 14]. Little research has been done about their role as early markers in diabetic patients.

This study was conducted to assess the urinary levels of NGAL in patients with type 2 diabetes mellitus to detect early kidney damage and correlate them with microalbuminuria.

Materials and Methods

This study was conducted in the Department of Biochemistry at Karpagam faculty of medical sciences and research. This study was approved by the Institutional Ethics Committee, Karpagam faculty of medical sciences and research, Coimbatore

Study design. Hospital based cross sectional study.

Sample size: 150 (calculated by 4pq/d2; where p is prevalence).

Study group. Patients with type 2 diabetes mellitus attending diabetic clinic at Karpagam Medical College during the study period.

Diabetes patients were classified into three groups according to their albumin/creatinine ratio (ACR) as follows. Group I (Normoalbuminuria): 50 diabetes patients with ACR < 30 mg/g of creatinine. Group II (Microalbuminuria): 50 diabetes patients with ACR 30–300 mg/g of creatinine. Group III (Macroalbuminuria): 50 diabetes patients with ACR > 300 mg/g of creatinine. Group IV (controls): 50 healthy subjects were selected as control group.

Inclusion criteria. Patients with type 2 diabetes mellitus (>5 yrs duration) according to American Diabetes Association (ADA) criteria.

Exclusion criteria. Patients with cardiovascular disorders (like CCF, H/o. CAD); pulmonary disorders (like respiratory failure); infections (Urinary tract infection) & inflammatory states; febrile illness; cancer; severe renal impairment (eGFR< 30 ml/min) and vigorous exercise.

Subjects who fulfilled the inclusion and exclusion criteria were included in the study. After explaining the nature of the study, written consent was obtained from all subjects before collecting blood sample.

Anthropometric measurements. Body mass was measured to the nearest 0.1 kg, with the participants dressed in light clothing. Barefoot standing height was measured to the nearest 0.1 cm with a wall-mounted stadiometer. Body mass index (BMI) is used for the assessment of fat distribution and obesity. Using standard measures of height and weight, BMI was measured using Quetelet's index (BMI = weight (kg)/height (m^2) [15].

Sample collection. First morning void urine samples (mid-stream urine) was obtained to measure urine albumin, microalbumin, creatinine and NGAL.

Two ml of whole blood was collected from the subjects in fasting state. Then the serum was separated after subjecting the collected blood to centrifugation at 2500 rpm for 5min at room temperature. The serum was divided into four aliquots and stored at -20°C for analysis of creatinine and fasting plasma glucose.

Estimation of biochemical parameters. Estimation of plasma glucose was done by glucose–oxidase method using fully automated analyser [16]. Estimation of creatinine was done by modified Jaffe's method using fully automated analyser [17]. Urine NGAL is estimated by a particle-enhanced turbidimetric immunoassay using Hitachi 917 analyzer. Microalbuminuria was estimated by immunoturbidimetry method using Hitachi 917 analyser. ACR was measured by urine albumin (mg/dl)/urine creatinine (mg/dl) × 1000.

Statistical analysis. Statistical Package for Social Sciences (SPSS, USA) was used to do the statistical analysis. All parameters were presented as mean ± SD. Student's *t*-test was used for comparing the means of continuous variables.

One-way analysis of variance (ANOVA) was done to compare the differences of parameters among various groups. Post hoc testing was done to

compare the difference among the studied groups. The correlations between various variables were calculated using the Pearsons correlation analysis. A receiver operating characteristic (ROC) analysis was done to calculate the area under the curve (AUC) and find the best diagnostic efficacy of each parameter. A linear regression analysis was performed to evaluate interrelationship between urinary NGAL, ACR and microalbuminuria. A $P < 0.05$ was taken as statistically significant.

Results

The comparison of demographic and baseline characteristics of the study population are shown in Table 1. Of 200 study participants, there were 50 normoalbuminuric with diabetes, 50 microalbuminuric with diabetes, 50 macroalbuminuric with diabetes, and 50 control populations. The anthropometric measures like height, weight, body mass index showed no statistically significant difference between the various diabetes groups and controls.

FBS were progressively higher among normoalbuminuria, microalbuminuria, andmacroalbuminuria. Systolic blood pressure was significantly higher in the marcoalbuminuria group compared to the diabetes patients with normoalbuminuria and normal healthy controls.

Urinary NGAL were significantly higher in the diabetes groups compared to the control group. Multiple comparisons indicated the significant difference in urinary NGAL values between the groups ($P < 0.05$) (Table 2). Intergroup comparison among three groups of diabetes patients showed that difference was significant with highest mean value in macroalbuminuria group and lowest in normoalbuminuria group.

Microalbumin was significantly high in diabetes patients with microalbuminuria and macroalbuminuria compared to the controls. Urinary ACR was significantly increased in diabetes patients with normoalbuminuria, microalbuminuria and macroalbuminuria compared to normal controls.

Table 1. Comparison of demographics and baseline characteristics among various groups

Variables	Group I	Group II	Group III	Control group	*P* Value
Age (years)	42.16 ± 3.96	43.02 ± 3.71	43.36 ± 3.65	43.18 ± 3.48	0.74
Height (m)	1.58 ± 0.08	1.56 ± 0.07	1.58 ± 0.08	1.57 ± 0.07	0.80
Weight (kg)	56.94 ± 6.00	56.52 ± 6.71	59.12 ± 6.38	56.04 ± 4.37	0.98
BMI (kg/m^2)	22.82 ± 2.04	23.03 ± 2.16	23.74 ± 2.62	22.76 ± 1.76	0.96
Systolic Bp (mm/Hg)	117.88 ± 6.64	119.44 ± 7.86	123.92 ± 5.75	111.68 ± 9.64	0.01*,‡
Diastolic Bp (mm/Hg)	71.36 ± 7.21	72.36 ± 7.98	83.80 ± 15.84	70.04 ± 6.93	0.00*
FBS(mg/dl)	96.44 ± 6.18	102.04 ± 9.69	123.44 ± 15.70	87.04 ± 9.30	0.001*,†,‡

Data are presented as mean ± SD. *$P \leq 0.05$ is statistically significant. ANOVA test was used to analyse the data. BMI – body mass index, FBS – fasting blood sugar, BP – blood pressure. *Significant difference between Group III and controls ($P < 0.05$). †Significant difference between Group III and Group II ($P < 0.05$). ‡Significant difference between Group III and Group I ($P < 0.05$)

Table 2. Comparison of clinical parameters among various groups

Parameters	Group I	Group II	Group III	Control group	*P* Value
Serum creatinine (mg/dl)	0.89 ± 0.17	1.25 ± 0.21	1.73 ± 0.32	0.83 ± 0.15	0.001*,†,‡
Microalbumin (mg/l)	18.96 ±7.66	91.76 ± 60.98	508.66 ± 156.56	14.04 ± 6.42	0.001*,†,‡
ACR (mg/g)	25.11 ±5.67	120.60 ±41.18	715.19 ± 143.21	6.64 ± 1.56	0.001*,†,‡
uNGAL (ng/ml)	41.57±14.84	97.32 ± 31.26	290.50 ± 92.98	12.26 ± 6.78	0.001*,†,‡

Data are presented as mean ± SD. $P \leq 0.05$ is statistically significant. ANOVA test was used to analyse the data. ACR – Albumin creatinine ratio, uNGAL – urinary neutrophil gelatinase associated lipocalin. *Significant difference between Group III and controls ($P < 0.05$). †Significant difference between Group III and Group II ($P < 0.05$). ‡Significant difference between Group III and group I ($P < 0.05$)

Association of urinary NGAL with microalbuminuria, ACR and serum creatinine in type 2 diabetes patients. Pearson's correlation was done to find the correlation of urinary NGAL with microalbuminuria, ACR and serum creatinine in patients with diabetes. The urinary NGAL showed good ($P < 0.001$) positive correlation with Microalbuminuria ($r = 0.876$), ACR ($r = 0.833$) and with serum creatinine ($r = 0.724$) (Table 3).

Linear regression analysis to find the effect of microalbuminuria on urinary NGAL and ACR among the diabetes patients. Linear regression analysis was performed to find the interrelationship between urinary NGAL, ACR and microalbuminuria. Regression analysis with urinary NGAL as dependent variable expressed a significant linear positive relationship with microalbuminuria (Table 4 and Fig. 1). Regression analysis with ACR as a dependent variable showed a significant relationship with microalbuminuria (Table 4 and Fig. 2).

Receiver operating characteristics curve analysis of urinary NGAL, microalbuminuria and ACR. Receiver operating characteristics (ROC) curve analysis was carried out to find the diagnostic efficacy of urinary NGAL, microalbuminuria and ACR in identifying the patients with DN (Table 5 and Fig. 3). The urinary NGAL showed a very good diagnostic profile, describing an AUC of 0.942 (95% CI: 0.902–0.981, $P < 0.001$) with a best cutoff value of 86.29 ng/ml (sensitivity 85%; specificity 72%). This is followed by ACR, showing an AUC of 0.855 (95% CI: 0.793–0.916, $P < 0.001$) and a best cutoff value of 114.6 (sensitivity 84%; specificity 79%). Microalbuminuria had an AUC of 0.874 (95% CI: 0.82–0.92, $P < 0.001$) and a best cutoff value of 72.4 mg/dl (sensitivity 82%; specificity 70%).

Table 3. Correlation analysis of NGAL with Microalbuminuria, ACR and serum creatinine in type 2 diabetes patients

Variables	*R* value	*P* Value
Microalbumin	0.876	0.001
ACR	0.833	0.001
Serum creatinine	0.724	0.001

Pearson correlation analysis was performed to analyze the data. *$P < 0.05$ is considered statistically significant. ACR – albumin creatinine ratio, uNGAL – urinary neutrophil gelatinase associated lipocalin

Discussion

Diabetic nephropathy is a metabolic syndrome characterized by presence of macroalbuminuria, a slow and progressive decline in glomerular filtration rate, elevated blood pressure, and increased cardiovascular mortality. Renal histopathologic changes in diabetes include glomerular basement membrane thickening, glomerular hypertrophy, mesangial cell expansion, podocyte injury, hyalinosis, and thickening of the afferent and efferent arterioles [18].

Diabetes patients are constantly exposed to hemodynamic and metabolic stress. Hemodynamic stress mainly leads to hypertension. Metabolic stress is closely related to hyperlipidemia and hyperglycemia. Hypertension, hyperglycemia and hyperlipidemia together leads to tubulointerstitial damage, endothelial dysfunction and atherosclerosis. Endothelial dysfunction progresses to albuminuria in patients with diabetes whereas tubular damage of the kidney leads to increase in the tubular biomarkers [19].

A study by Yaqoob M et al. showed that tubular injury and endothelial dysfunction may precede the onset of microalbuminuria in patients with diabetes mellitus. Diabetes patients with even normal eGFR are at greater risk for AKI compared to normal healthy subjects [20]. Renal tubular markers such as NGAL, MCP (Monocyte chemoattractant protein-1), KIM-1 (Kidney injury molecule 1), urinary liver

Table 4. Linear regression analysis to find the effect of Microalbuminuria on urinary NGAL and ACR among the diabetes patients

Independent variables	Nonstandardised coefficients B value	Standardised coefficients β	R square value	P value
uNGAL	0.488	0.876	0.767	0.001
ACR	1.162	0.866	0.749	0.001

*$P < 0.05$ is considered statistically significant. ACR – albumin creatinine ratio, uNGAL – urinary Neutrophil gelatinase associated lipocalin

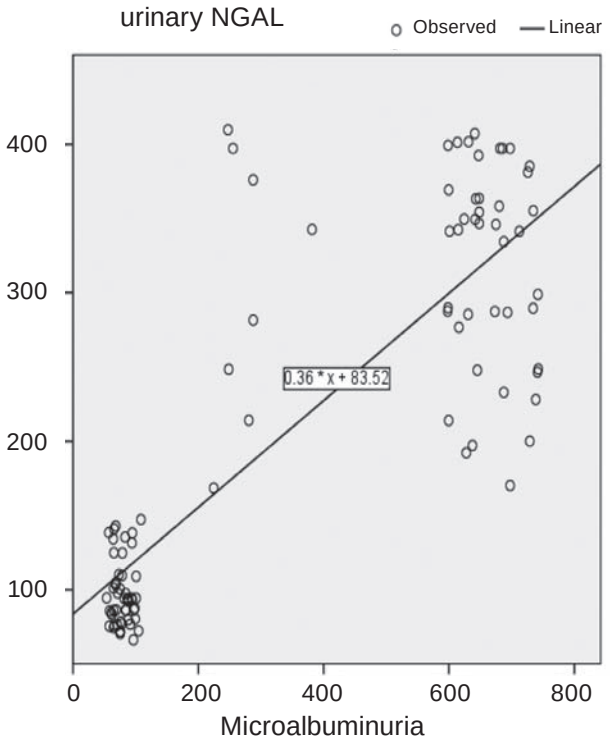

Fig. 1. Linear regression analysis between microalbuminuria and urinary NGAL among the diabetes cases

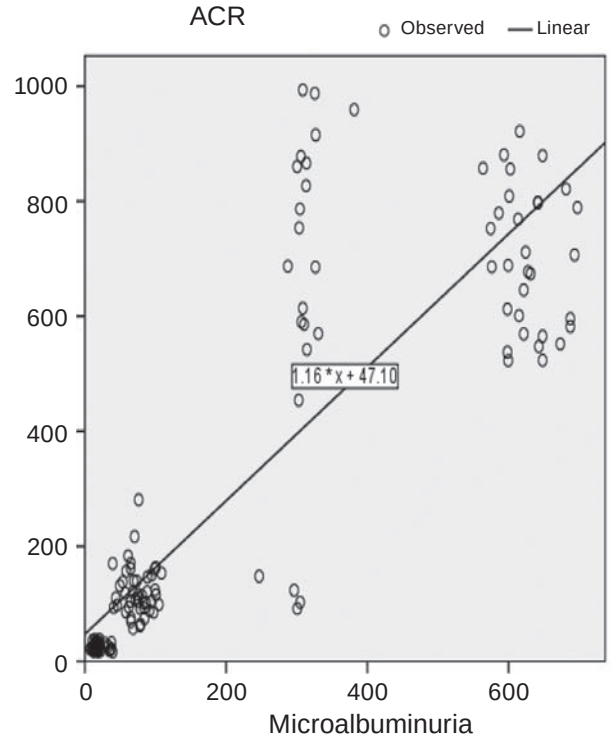

Fig. 2. Linear regression analysis between microalbuminuria and ACR among the diabetes cases

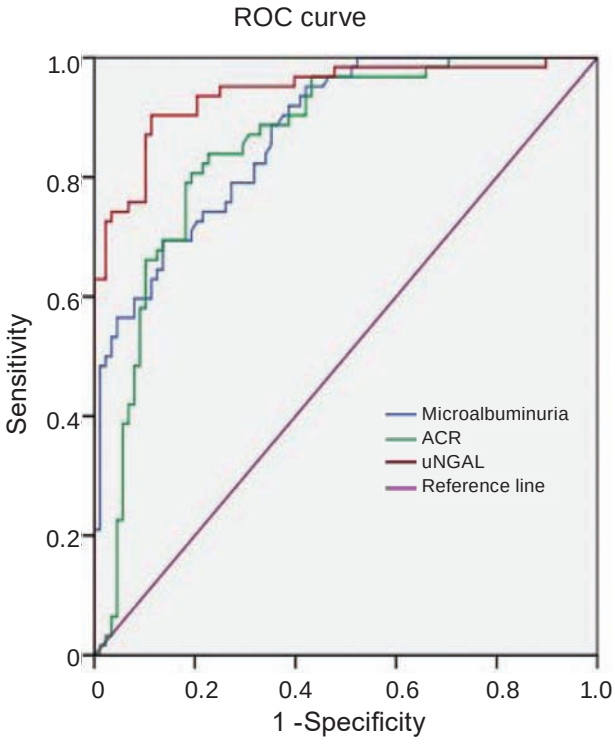

Fig. 3. ROC curve of urinary NGAL, Microalbuminuria, and ACR

type fatty acid binding protein (uLFABP) are used to predict the onset and progression of DN [21-24].

NGAL is a 25 kDa protein consisting of a polypeptide chain of 178 amino acids and belongs to the 'lipocalin' superfamily. It is expressed by neutrophils and various epithelial cells. This protein is released in blood and urine following ischemic and nephrotoxic injury from the tubular cells. Variable degrees of NGAL gene expression is demonstrated in human tissues like uterus, prostate, salivary glands, lung, trachea, stomach, colon, and kidney [25].

NGAL is currently considered as one of the most promising biomarkers in clinical nephrology and has been extensively studied in acute kidney disease. A few studies have demonstrated that NGAL might also be elevated in some other conditions other than renal injury. Therefore, we have excluded those patients with infection, neoplasia and inflammation [25, 26].

High glucose levels and diabetic substrates, including advanced glycation end-products, carbonyl intermediates, and growth factors, promote renal tubular hypertrophy and fibrosis. Therefore, tubular biomarkers may be crucial as glomerular markers

Table 5. Receiver operating characteristics curve analysis of urinary NGAL, Microalbuminuria, and ACR

Variables	Area	Standard error	Asymptotic Sig	Asymptotic 95% confidence interval	
				Lower Bound	Upper Bound
uNGAL	0.942	0.020	0.001*	0.902	0.981
ACR	0.855	0.031	0.001*	0.793	0.916
Microalbumin	0.874	0.027	0.001*	0.821	0.927

*$P < 0.05$ is considered statistically significant. ACR – albumin creatinine ratio, uNGAL – urinary Neutrophil gelatinase associated lipocalin

for early diagnosis of renal impairment in patients with diabetes [27, 28]. As NGAL is a marker of distal tubular injury, it is not going to be affected by albuminuria or proteinuria, which suggests that tubular injury may occur before glomerular injury in patients with type 2 diabetes [10]. Markers of tubular injury predicting onset of albuminuria and progression of diabetes have been observed in various other studies using other biomarkers such as uLFABP, MCP, KIM-1 [22-24].

But a study by Kim et al. suggested that there was no significant difference in NGAL levels among normoalbuminuria, microalbuminuria and control group which contradicts the role of NGAL as an early biomarker of DN [29].

In our study, the urinary NGAL levels were significantly elevated in microalbuminuria and macroalbuminuria diabetes patient groups when compared to the control. Urine NGAL showed a positive correlation relationship with microalbuminuria in diabetic patients which indicates urinary NGAL was positively correlated with the progression of DN.

Bolignano et al found that serum and urinary NGAL were significantly elevated in diabetic patients compared to the control group and that NGAL levels were elevated in diabetic patients without early signs of glomerular damage [30].

Nielsen et al reported that elevated urine NGAL in type 1 diabetic patients with or without albuminuria indicates tubular damage [24]. A study by Jiao et al showed increased levels of NGAL in both serum and urine, which correlated with the severity of renal damage in patients with diabetes mellitus. He also suggested that NGAL is elevated both in serum and urine, even before albumin appears in urine [31].

Nauta et al showed that NGAL is increased in diabetes patients with normoalbuminuria when compared to the controls [10]. Lacquaniti et al. suggested that NGAL is elevated in diabetes patients even before diagnosis of albuminuria and hence NGAL can be used for the detection of early kidney involvement of normoalbuminuric DN [32].

Zachwieja et al. found that urine and serum NGAL were significantly increased in diabetes children with normoalbuminuria, and also suggested that normoalbuminuria does not exclude DN [33].

Normoalbuminuric diabetes patients had significantly greater mean urinary NGAL levels than the controls which suggests that tubular injury may occur before the onset of glomerular injury in patients with diabetes. This suggests that tubular dysfunction is due to hemodynamic and metabolic stress which is secondary to chronic hyperglycemia in patients with diabetes mellitus [34, 35].

This finding supports the hypothesis that urine NGAL can be used as a marker for the early detection of diabetic nephropathy. Besides, the mean value of urine NGAL was also observed to be increased according with the degree of renal impairment. This finding indicates that urine NGAL can also be used in determining the severity of renal disease.

ROC curve analysis was done to compare the diagnostic performance of NGAL, microalbuminuria and ACR and to find which is more sensitive and specific in the diagnosis of DN. Urinary NGAL had a higher diagnostic performance of diabetic nephropathy compared to ACR and microalbuminuria.

Microalbuminuria is considered as the earliest marker of development of diabetic nephropathy and is usually associated with significant glomerular damage. However, recent studies showed that microalbuminuria does not necessarily reflect permanent renal impairment. In addition, several studies suggest that early structural damage in both glomerular and tubular compartments may be even present in subjects with normoalbuminuria.

Urinary NGAL measurement is more sensitive than microalbumin and can be a useful tool for evaluating early renal involvement in patients with diabetes.

Limitations of the study. Major limitation of our study was small sample size. It is a single centre study with cross-sectional design. We did not collect 24 hour urine sample due to technical difficulties in collecting sample.

Conclusion. Urinary NGAL has a positive association with microalbuminuria and it can be a noninvasive tool for diagnosis and monitoring the progression of diabetic nephropathy. Urinary NGAL measurement is more sensitive than microalbumin and can be used as an important tool for detecting early renal involvement in patients with diabetes mellitus even earlier to incipient nephropathy.

Conflict of interest. Authors have completed the Unified Conflicts of Interest form at http://ukrbiochemjournal.org/wp-content/uploads/2018/12/coi_disclosure.pdf and declare no conflict of interest.

НЕЙТРОФІЛЬНИЙ ЖЕЛАТИНАЗО-АСОЦІЙОВАНИЙ ЛІПОКАЛІН СЕЧІ, ЯК БІОХІМІЧНИЙ МАРКЕР МІКРОАЛЬБУМІНУРІЇ ДЛЯ РАННЬОГО ВИЯВЛЕННЯ НЕФРОПАТІЇ У ПАЦІЄНТІВ ІЗ ЦУКРОВИМ ДІАБЕТОМ 2 ТИПУ

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Діабетична нефропатія є одним із мікросудинних ускладнень цукрового діабету. Метою дослідження було оцінити діагностичну цінність рівня нейтрофільного желатиназо-асоційованого ліпокаліну (NGAL) у сечі як біомаркера для раннього виявлення нефропатії у пацієнтів із цукровим діабетом 2 типу. У дослідженні взяли участь 150 пацієнтів із діабетом 2 типу, яких розділили на 3 рівні групи відповідно до альбумін/креатинінового співвідношення в сечі (ACR), у тому числі пацієнти з нормоальбумінурією (ACR < 30 мг/г креатиніну), мікроальбумінурією (ACR = 30-300 мг/г креатиніну) та макроальбумінурією (ACR > 300 мг/г креатиніну). До контрольної групи було відібрано 50 здорових осіб. NGAL у сечі визначали методом турбодиметричного імуноаналізу на аналізаторі Hitachi 917. Також оцінювали рівень глюкози та креатиніну у сироватці крові. Виявлено, що рівень NGAL у сечі був значно підвищений у групах хворих на діабет із мікроальбумінурією та макроальбумінурією у порівнянні з контрольною групою та групою хворих на діабет з нормоальбумінурією. Рівень NGAL у сечі позитивно корелював із мікроальбумінурією у пацієнтів із діабетом. Показано, що визначення NGAL у сечі пацієнтів із діабетом має вищу діагностичну цінність для раннього виявлення діабетичної нефропатії у порівнянні з визначенням альбумін-креатинінового співвідношення та мікроальбумінурією.

Ключові слова: желатиназо-асоційований ліпокалін, діабетична нефропатія, альбумін/креатинінове співвідношення, мікроальбумінурія.

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