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Plasma type IV collagen levels as a potential biomarker for early detection of nephropathy in diabetes mellitus patients

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Abstract

Diabetic nephropathy represents a microvascular complication related to type 2 diabetes mellitus (T2DM) that ultimately causes end-stage renal disease. Our study aimed to evaluate the association of plasma type IV collagen with albuminuria status and to assess the clinical significance of plasma type IV collagen as a potential biomarker in the early stage of diabetic nephropathy. The study comprised 75 participants diagnosed with T2DM allocated equally (n=25) into three groups: (A) normal albuminuria levels, (B) microalbuminuria, and (C) macroalbuminuria, depending on their urine albumin-to-creatinine ratio. A comparative analysis was conducted between these groups and a control group (D, n=15). The enzyme-linked immunosorbent assay (ELISA) method was employed for measuring plasma type IV collagen levels. The results revealed that plasma type IV collagen levels were significantly higher in T2DM groups than in the control group. Moreover, diabetic patients without albuminuria had significantly higher plasma type IV collagen levels than the control group ($p<0.001$). Furthermore, albuminuria levels among diabetic patient groups were significantly increased as albuminuria categories increased ($p<0.001$). A significant positive correlation existed between plasma type IV collagen and glycated hemoglobin (HbA1c) levels in the macroalbuminuric diabetic group. Our study employed the receiver operating characteristic (ROC) curve analysis to determine plasma type IV collagen diagnostic utility in macroalbuminuria prediction. The ROC curve analysis revealed that type IV collagen can significantly determine macroalbuminuric patients at a cutoff value of 2.25 with sensitivity, specificity, positive predictive value, and negative predictive value of 68%, 100%, 100%, and 75.8%, respectively ($p<0.001$). In conclusion, plasma type IV collagen levels might serve as a valuable predictor of albuminuria onset in patients with T2DM.

Keywords: Plasma type IV collagen; Nephropathy; Diabetes mellitus; Biomarke

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Introduction

Diabetic nephropathy (DN) can be defined as a microvascular disorder characterized by the gradual deterioration of glomerular filtration that results in end-stage renal disease (ESRD).[1] In 2021, the estimated global prevalence of diabetes mellitus (DM) among people with 20–79 years of age was 11%, which is expected to increase to 12% by 2045.[2] Worldwide, about

40%–50% of DN results when diabetes damages blood vessels and other cells of the kidneys.[3] In addition, the initial detection of renal impairment in type2 DM (T2DM) patients is commonly recognized through microalbuminuria.[4] Microalbuminuria substantially correlates with the complex histopathological picture of glomerular and tubular damage; therefore, it probably represents a nonspecific indicator of ongoing renal injury.[5] Individuals diagnosed with T2DM can develop renal impairment despite maintaining normoalbuminuria.[6]

Type IV collagen is the predominant protein component of the basement membrane, which constructs a network beneath the epithelial and endothelial cells.[7] This network acts as a barrier, effectively segregating several tissue constituents.[8] The glomerular basement membrane and mesangial matrix have a significant abundance of type IV collagen. Type IV collagen possesses significant signaling capabilities because subdomains, including tumstatin, are released when special proteases degrade the protein.[9] Identifying this particular protein in physiological fluids is thought to be the first indication of DN onset during its early stages.[10] Therefore, it is essential to devise more sensitive indicators for identifying the early stage of DN.[11] This study aimed to assess plasma type IV collagen levels in different diabetic patients accompanied by different stages of albuminuria and its clinical significance as a biomarker during the early stage of DN. Additionally, we aimed to identify the possible correlation between plasma type IV collagen levels and lipid profile, kidney function tests, glycated hemoglobin, and urine albumin-to-creatinine ratio (ACR).

Subjects and Methods

This was a case-control study that included 75 patients diagnosed with diabetes, selected from the Internal Medicine Outpatient Clinic at Al-Zahraa Hospital in Egypt during the period from December 2021 to June 2022.

The inclusion criteria included patients aged ≥45 years of both genders with a confirmed diagnosis of T2DM and maintained on oral hypoglycemic medications for a duration exceeding five years. The exclusion criteria included the following: patients with a history of hypertension, atypical urine sediment, urinary tract infections, other renal diseases, neoplastic disorders, acute myocardial infarction, stroke, and chronic liver diseases.

The study participants were categorized equally into three groups (n=25 subjects each) based on their urine ACR. Group (A) included 25 diabetic patients with normoalbuminuria (<30 mg/g creatinine), group (B), 25 diabetic patients with microalbuminuria (30–299 mg/g creatinine), and group (C), 25 diabetic patients with macroalbuminuria (≥ 300 mg/g creatinine). The groups were comparable for age and sex with the control group (group D), consisted of 15 apparently healthy subjects.

Demographic and clinical data were obtained and subsequently maintained confidentially following the admission of the participants. Full history was recorded, and physical examinations were conducted, which included body weight, height, waist-to-hip circumference and blood pressure including systolic and diastolic blood pressure readings. The body mass index (BMI) was calculated by dividing the weight (kg) by the square of height (m^2).

Blood sampling and measurement of studied parameters

Under complete aseptic condition and after 8 hours fasting, a venous blood sample (5 mL) was obtained from each study subject. Blood samples were collected into two tubes. The first tube contained 3 mL on EDTA and used for estimation of glycated hemoglobin (HbA1c) and for plasma separation, stored at -80 ℃ until used. The second was a plain tube contained 2 mL blood, allowed to coagulate and serum was separated by centrifugation. The separated serum was used for estimation of kidney function tests and fasting blood glucose.

The HbA1c and kidney function tests were performed using the automated clinical chemistry analyzer (Cobas c311, Roch diagnostic, Germany), according to the manufacturer's instructions. Blood glucose (fasting and post-prandial) was assessed by

glucose kits (LOT GLUCO0102023-2, liquizyme, Germany), and measured through a spectrophotometer (ELICO UV-Vis™ spectrophotometer SL 159, version 6.1, India).

Another venous blood sample (2 mL) was collected after 10 hours fasting in a plain tube and used for lipid profile using enzymatic kits (Ensure Biotechprivate limited™, India) and read by spectrophotometery. Finally, a blood sample (2 mL) was collected (2 hr after the meal) in a plain tube for assessment of post-prandial glucose. A morning urine sample was collected and used for measurement of ACR by assesing urine albumin and creatinine using a clinical chemistry analyzer (Cobas c311, Roch diagnostic, Germany).

Assessment of type IV collagen plasma levels

Plasme type IV collagen was measured by enzyme-linked immunosorbent assay (ELISA) Kits (Catalogue No 201-12-1381, SunRed, China), according to the manufacturer's instructions. The final ELISA products were measured by a microtiter reader (Biokit ELISA Reader ELX800, USA).

Statistical Analysis

Statistical analysis was conducted through the statistical package for the social sciences (SPSS) program (version 26.0, IBM Corporation, Armonk, New York). Our study employed the chi-square test, analysis of variance (ANOVA) or F test, Kruskal-Wallis test, correlation analysis utilizing Spearman's approach, multiple linear regression analysis used to identify the most important factors significantly affecting plasma type IV collagen level and receiver operating characteristic (ROC) curve analysis. The Kolmogorov-Smirnov test was conducted to determine the adequacy of the sample distribution. Categorical data are reported as numerical and percentage,while continuous data are reported as mean and standard deviation (SD). The threshold of 0.05 was regarded as a significant difference.

Results

Table 1 lists the demographic and clinical data of all participants. The results revealed a statistically significant variation in BMI, waist-to-hip circumference, SBP, and DBP across the four groups. However, gender, age, and DM duration the groups exhibited nonsignificant differences in.

Table 1. Comparison of demographic and clinical data of the study participants.

Parameters		Group (A) (n = 25)		Group (B) (n = 25)		Group (C) (n = 25)		Group (D) Controls (n = 15)		*p*-value
		No.	%	No.	%	No.	%	No.	%	
Gender	Male	12	48.0	12	48.0	10	40.0	6	40.0	NS ^{X2}
	Female	13	52.0	13	52.0	15	60.0	9	60.0	
Age (years)										
Mean ± SD		61.20 ± 6.51		64.36 ± 9.84		60.28 ± 8.94		56.13 ± 5.83		
Median		60.0		65.0		60.0		55.0		NS*
Range		50.0–70.0		47.0–80.0		45.0–80.0		45.0–65.0		
		Mean ± SD		Mean ± SD		Mean ± SD		Mean ± SD		
Duration (years)		8.16 ± 1.77		9.76 ± 3.28		8.16 ± 2.44		0.00 ± 0.00		*NS
Body mass index (Kg/m^2)		28.57 ± 0.92		27.40 ± 1.14		28.14 ± 1.52		25.11 ± 1.11		< 0.001$^{\#}$
Waist-to-hip circumference		0.88 ± 0.02		0.90 ± 0.02		0.92 ± 0.02		0.91 ± 0.02		< 0.001*
Systolic blood pressure (mm/Hg)		116.80 ± 13.14		122.40 ± 13.00		128.80 ± 12.36		113.33 ± 6.99		0.001*
Diastolic blood pressure (mm/Hg)		75.20 ± 7.70		78.00 ± 7.64		80.40 ± 6.76		75.00 ± 5.00		0.045*

SD: standard deviation, X^2: Chi-Square test, *KW: Kruskal-Wallis test, and $^{\#}$:One Way ANOVA test.
$p > 0.05$ is not significant (NS).

The levels of fasting blood glucose, postprandial glucose, HbA1c, serum cholesterol, serum triglycerides, serum creatinine, low-density lipoprotein, urine albumin-creatinine ratio and blood urea were significantly higher in the diabetic group than in the control group and increased with the progression of albuminuria categories (p<0.001, for all). However, high-density lipoprotein levels were lower in the diabetic patient groups than in the control group (p<0.001; Table 2).

Table 2. Comparison of laboratory data among the studied groups.

Parameters	Group (A) (n = 25)	Group (B) (n = 25)	Group (C) (n = 25)	Group (D) (n = 15)	*p*-value
	Mean ± SD	Mean ± SD	Mean ± SD	Mean ± SD	
Fasting blood glucose (mg/dL)	137.46 ± 8.32	150.36± 12.88	230.96± 21.62	78.53± 5.90	< 0.001*
Post-prandial glucose (mg/dL)	177.66 ± 6.40	225.96± 17.40	335.52± 29.61	113.13± 3.36	< 0.001*
Glycated hemoglobin (%)	7.69± 0.34	9.07± 0.32	10.68± 0.33	5.46± 0.26	< 0.001*
Serum cholesterol (mg/dL)	203.22± 14.25	217.60± 11.17	231.04± 11.27	169.65± 8.29	< 0.001#
Serum triglycerides (mg/dL)	143.20± 8.90	145.72± 11.06	189.92± 17.80	91.03± 5.80	< 0.001*
Low-density lipoprotein (mg/dL)	121.08± 6.87	128.96± 11.92	141.76± 14.79	101.07± 6.59	< 0.001*
High-density lipoprotein (mg/dL)	41.88± 8.00	40.40± 1.28	39.06± 1.92	47.96± 2.50	< 0.001*
Urine albumin creatinine ratio (%)	15.78± 6.51	181.70± 70.83	583.2 6± 226.1	12.77± 5.69	< 0.001*
Blood urea (mg/dL)	28.74± 2.12	32.64± 2.88	38.88± 5.13	24.75± 1.60	< 0.001*
Serum creatinine (mg/dL)	0.82± 0.07	1.08± 0.15	1.19± 0.18	0.80± 0.06	< 0.001*

*$p \leq 0.05$ is significant.

The study revealed a significant elevation in plasma type IV collagen levels among normoalbuminuric diabetic patients when compared with the control group (Figure 1, and Table 3). Moreover, their levels exhibited a significant rise as the categories of albuminuria increased in subjects with diabetes (p< 0.001).

Table 3. Comparison of plasma type IV collagen between the studied groups.

Parameter		Group (A) (n = 25)	Group (B) (n = 25)	Group (C) (n = 25)	Group (D) (n = 15)	*p*-value
Plasma type IV collagen	Mean ± SD	4.18 ± 5.10	9.08 ± 3.71	15.69 ± 12.47	1.76 ± 0.49	< 0.001KW
	Median	1.84	3.71	14.25	1.41	
	Range	1.29–21.26	1.16–31.30	1.89–54.50	0.51–2.25	

KW: Kruskal-Wallis test *$p \leq 0.05$ is significant.

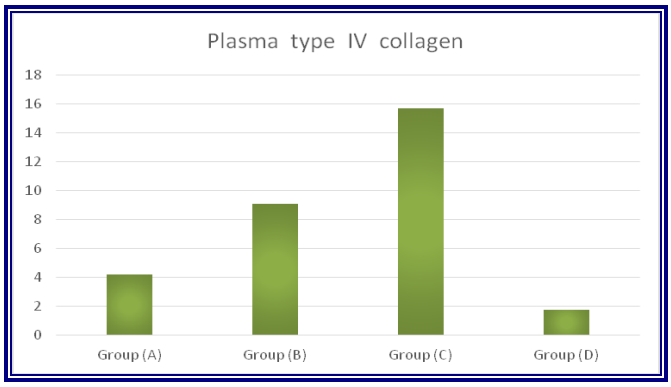

Figure 1. Difference in plasma type IV collagen between the study groups.

The results showed that plasma type IV collagen had a significant positive correlation with the blood urea in group A (r = 0.421, *p*=0.036; Figure 2) and HbA1c in group C (r = 0.396, *p*=0.05; Figure 3) while had a significant negative correlation with post-prandial blood glucose in group B (r = –0.444, *p* = 0.026; Figure 4).

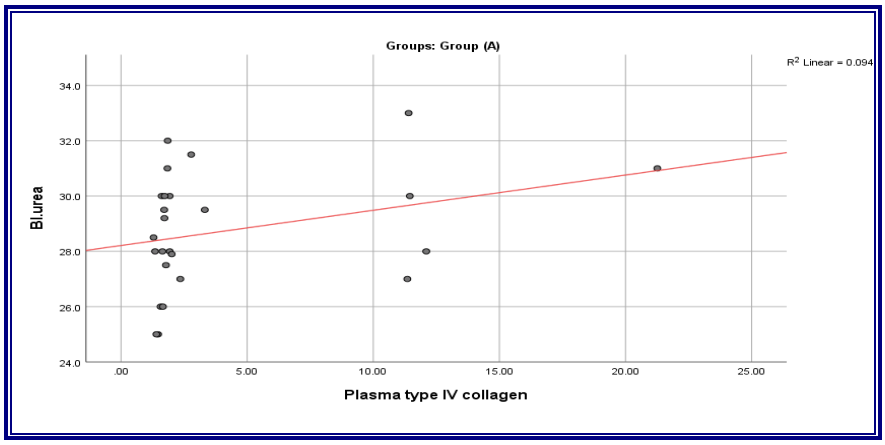

Figure 2. A scatter plot showing a positive correlation between plasma type IV collagen and blood urea in group A.

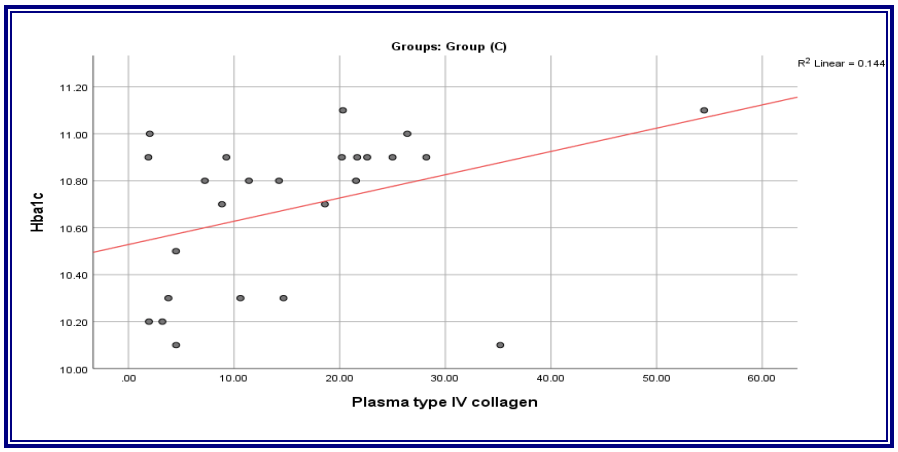

Figure 3. A scatter plot showing a positive association between plasma type IV collagen and HbA1c in group C.

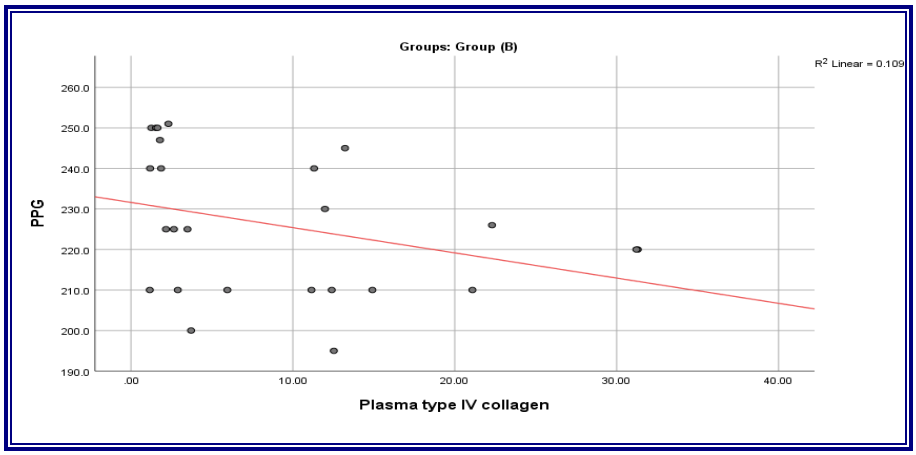

Figure 4. A scatter plot showing a negative correlation between plasma type IV collagen and post-prandial blood glucose in group B.

The results of multiple linear regression analysis (Table 4) indicated that the most important factors significantly affecting plasma type IV collagen level were urine ACR (p=0.044) and LDL (p=0.026).

Table 4. Multiple linear regression analysis of the factors affecting plasma type IV collagen levels.

	Unstandardized Coefficients		Standardized Coefficients Beta	p-value
	B	Standard error		
Urine albumin creatinine ratio	0.012	0.006	0.331	0.044
Fasting blood glucose	0.065	0.056	0.349	NS
Post-prandial glucose	– 0.048	0.040	– 0.388	NS
Glycated hemoglobin	– 0.098	1.904	– 0.018	NS
Serum cholesterol	0.080	0.079	0.189	NS
Serum triglycerides	– 0.072	0.069	– 0.250	NS
Low-density lipoprotein	0.192	0.085	0.332	0.026
High-density lipoprotein	– 0.061	0.197	– 0.032	NS
Blood urea	0.024	0.270	0.014	NS
Serum creatinine	6.505	6.251	0.137	NS

$p > 0.05$ is not significant (NS).

The ROC curve analysis results (Table 5) demonstrated that plasma type IV collagen could significantly determine normoalbuminuric patients at a cutoff value of 1.41 with sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) of 88%, 53.3%, 65.3%, and 81.6%, respectively (p = 0.002; Figure 5). Plasma type IV collagen can significantly determine patients with microalbuminuria at a cutoff value of 2.25 with sensitivity, specificity, PPV, and NPV at 68%, 100%, 100%, and 75.8%, respectively (p<0.001; Figure 6). Furthermore, plasma type IV collagen can significantly determine macroalbuminuric patients at a cutoff value of 2.25 with sensitivity, specificity, PPV, and NPV of 68%, 100%, 100%, and 75.8%, respectively (p<0.001; Figure 7).

Table 5. The validity of collagen type IV in predicting albuminuria among the studied groups.

Parameter	Cutoff value	AUC	Sensitivity	Specificity	PPV	NPV	*p*-value
Plasma type IV collagen (group A)	> 1.41	0.753	88%	53.3%	65.3%	81.6%	0.002
Plasma type IV collagen (group B)	> 2.25	0.853	68%	100%	100%	75.8%	< 0.001
Plasma type IV collagen (group C)	> 2.25	0.980	88%	100%	100%	89.3%	< 0.001

PPV= Positive Predictive Value, NPV= Negative Predictive Value, AUC= Area under Curve

*$p \leq 0.05$ is significant.

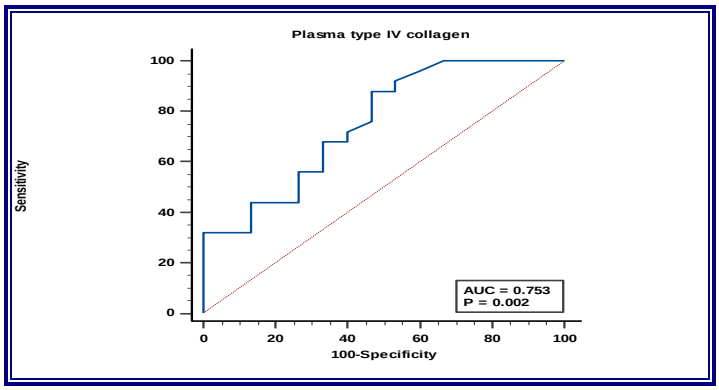

Figure 5. The diagnostic value of plasma type IV collagen in predicting normoalbuminuria.

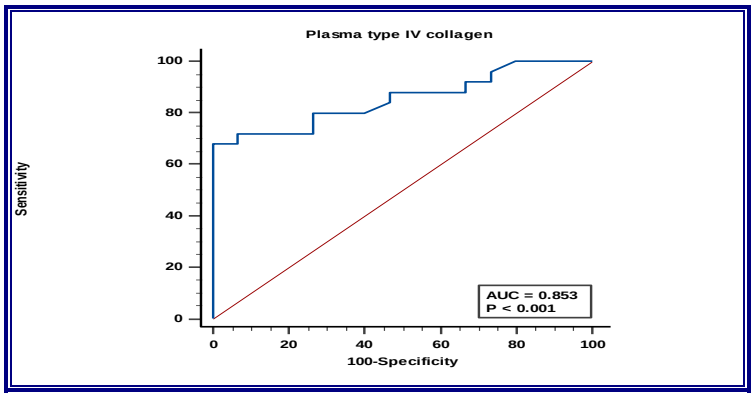

Figure 6. The diagnostic value of plasma type IV collagen in predicting microalbuminuria.

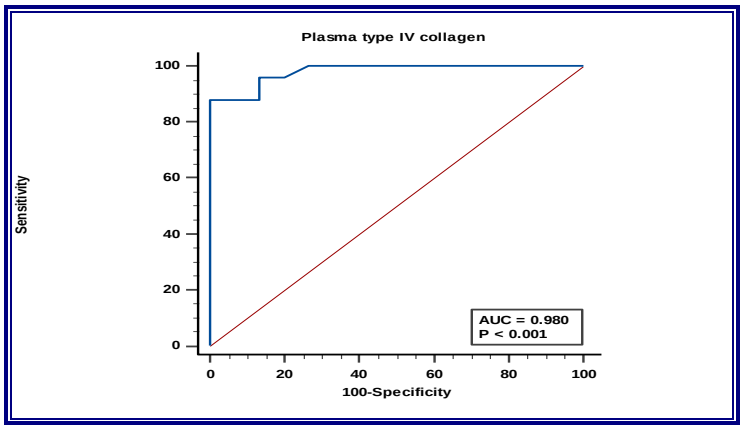

Figure 7. The diagnostic value of plasma type IV collagen in predicting macroalbuminuria.

Discussion

DN is a microvascular complication that occurs in around 20%–40% of patients diagnosed with T2DM and is clinically characterized by persistent albuminuria and a progressive decline in renal function, which eventually results in ESRD.[12] Although microalbuminuria in diabetic patients is considered to be the best predictor of progression to ESRD and cardiovascular events, more sensitive and specific markers of kidney damage might help to diagnose and treat DN at an earlier stage to prevent the progression to renal failure. Type IV collagen is a protein with three alpha polypeptides which serves as the main basement membrane constituent of the glomerulus, tubules, and mesangial matrix. Increased type IV collagen expression in renal glomeruli or tubules has been indicated to mark an advanced stage of kidney function loss in T2DM.[13] Therefore, we aimed to assess the correlation of plasma type IV collagen with albuminuria in diabetic patients and its clinical significance as a biomarker in the early stages of DN.

Our study found no variation in age, sex, and disease duration among all studied groups (p=0.017), which is consistent with the results of a study by Omran et al., 2022.[14] Contrary to our findins, Hamid et al., 2021[15] detected significant changes in values of disease duration in diabetic groups compared to the control group.

Our study demonstrated a significant variation in BMI and waist-to-hip circumference between the normoalbuminuric, microalbuminuria, and macroalbuminuric groups compared with the control group. The waist-to-hip circumferences of the microalbuminuria and macroalbuminuria groups were significantly less than in the normoalbuminuric and control groups; this was in accordance with a study carried out by Martin et al., 2020[16] in which there was a significant positive correlation between increasing BMI and increasing proteinuria amongest obese patients with diabetic kidney diseases. This may be due to changes in renal endothelial function and hemodynamics caused by the reaction of insulin resistance through the production of adipokines and growth factors.These effects include oxidative stress, inflammation, activation of the renin angiotensin aldosterone system, abnormal lipid metabolism, and insulin resistance. This may lead to ectopic accumulation of lipids and increases in renal sinus fat, glomerular hypertension, increased permeability of the glomeruli, hyperfiltration, and consequently focal or segmental glomerulosclerosis.[16]

Additionally, we demonstrated a statistically significant increase in the average SBP and DBP among patients in the macroalbuminuric group. In accordance with our findings, the study by Mahendran et al., 2016[11] reported a significant elevation in SBP among DM individuals compared to healthy control individuals. However, this disagree with data reported by a study of Hamid et al., 2021.[15] The possible explanation might be that co-morbid hypertension is considered risk factor for DN, leading to glomerular hypertension and the activation of mediators that induce inflammation, fibrosis, and further injury of the glomeruli.[17]

The normoalbuminuric, microalbuminuria and macroalbuminuric diabetic patients demonstrated significantly elevated fasting blood glucose levels compared to the control group (p<0.001). The diabetic patients who exhibited either microalbuminuria or macroalbuminuria demonstrated significantly elevated post-prandial blood glucose levels and HbA1c compared to the control group. Moreover, their levels exhibited a rise in accordance with the rising categories of albuminuria observed in patients with DM. Consistent with our findings, the study by Mahfouz et al., 2020[18] demonstrated that the average fasting blood galucose and HbA1c levels in individuals in all three DM groups showed a significant elevation compared to the control group (p =0.05). The explanation of such finding is that poor hyperglycemic control leads to the elevated attachment of glucose molecules to the hemoglobin in the erythrocyte and this leads to an increase in HbA1c with consequent complications such as nephropathy.[19]

Our study found that normoalbuminuric diabetic patients had significantly higher serum cholesterol, urine ACR, serum creatinine, and blood urea levels than the control group. Moreover, their levels exhibited a rise in accordance with the rising of albuminuria categories in diabetic patients (p<0.001). A possible mechanism for these findings is that the changes in renal endothelial function and hemodynamics may be caused by the reaction of insulin resistance. Furthermore, the secretion of inflammatory factors from central or visceral adipose tissue, such as tumor necrosis factor-alpha and interleukin-6, causes endothelial dysfunction at the glomerular level,increasing urine albumin excretion.[20]

In the present study, we observed a significant elevation in plasma type IV collagen levels among diabetic and normoalbuminuric diabetic patients when compared with the control group (p<0.001) in accordance with the rising albuminuria categories in diabetic patients (p<0.001). In the same line, the study by Mahendran et al., 2016[11] demonstrated that normoalbuminuric diabetic patients had significantly increased levels of type IV collagen in both plasma and urine compared with the control group. In addition, our results agreed with those reported by Mahfouz et al., 2020 [18] who demonstrated a significant elevation in type IV collagen levels in normoalbuminuric, microalbuminuria, and macroalbuminuric diabetic groups compared to the control group. Moreover, the microalbuminuria and macroalbuminuric diabetic groups had a higher level of type IV collagen than the normoalbuminuric group. This could be explained by the pathogenetic mechanism that hyperglycemia stimulates type IV collagen production by activating the cellular transforming growth factor beta. This leads to malfunction, podocyte injury and deposition of proteins in the extracellular matrix of the nephron with albumin leak and apoptosis.[21]

Our results demonstrated that in normoalbuminuric patients, plasma type IV collagen had a significant positive correlation with blood urea but a significant negative correlation with high-density lipoprotein. However, the study by Hamid et al., 2021[15] revealed that the plasma type collagen IV was negatively correlated with serum creatinine in normoalbuminuria, which was inconsistent with our results.

In our study, microalbuminuria patients had a significant positive correlation of plasma type IV collagen with urine ACR but a significant negative correlation to post-prandial blood glucose, which aligns with the results of Mahfouz et al., 2020.[18] Meanwhile, in macroalbuminuric patients, plasma type IV collagen exhibited a significant positive correlation with HbA1c, which contracted with the findings represented by Mahfouz et al., 2020.[18]

Based on the multiple linear regression analysis results, the most important factors significantly affecting the plasma type IV collagen level were urine ACR (p=0.044) and LDL (p=0.026). These outcomes agreed with the results of Mahendran et al., 2016[11] who demonstrated a significant correlation between plasma and urinary type IV collagen levels with ACR in diabetic patients with microalbuminuria and macroalbuminuria.

The ROC curve analysis was performed to measure plasma type IV collagen diagnostic efficacy for the prediction of normoalbuminuric. The analysis showed that at a cutoff value of 1.41, the sensitivity, specificity, PPV, and NPV were 88%, 53.3%, 65.3%, and 81.6%, respectively (p=0.002). A study by Hamid et al., 2021[15] reported that in the normoalbuminuric group, the AUC was 0.91, and the optimal cutoff point determined from the ROC curve was identified at 7.5 ng/mL, with a sensitivity and specificity of 92% and 89.3%, respectively.

Moreover, our ROC curve analysis results of plasma type IV collagen diagnostic significance in the prediction of microalbuminuria indicated that at a cutoff value of 2.25, the sensitivity, specificity, PPV, and NPV were 68%, 100%, 100%, and 75.8%, respectively (p<0.001). This agreed with those of the study by Mahfouz et al., 2020[18] who revealed that to discriminate normoalbuminuric diabetic patients from microalbuminuria diabetic patients, the cutoff value for type IV collagen was 2.6 ng/mg, and AUC was 0.916. Thse results indicated the good validity of the type IV collagen to differentiate

normoalbuminuric diabetic patients from microalbuminuria diabetic patients.

Additionally, our ROC curve analysis was conducted to asses plasma type IV collagen diagnostic efficacy in predicting macroalbuminuria, revealing that at a cutoff value of 2.25, the sensitivity, specificity, PPV, and NPV were 68%, 100%, 100%, and 75.8%, respectively (p<0.001). Such data are consistent with those reported by the study of Mahfouz et al., 202018 who demonstrated that to discriminate normoalbuminuric from macroalbuminuric diabetic patients, the cutoff values for type IV collagen was 2.7 ng/mg and AUC of 0.964.

These results indicated the good validity of the type IV collagen to differentiate normoalbuminuric from macroalbuminuric diabetic patients. Furthermore, the study by Hamid et al., 2021[15] revealed that collagen IV in the macroalbuminuric group of patients showed an excellent ability to predict macroalbuminuria in diabetic patients from those without any disease, with high sensitivity and specificity (≥90%), in terms of prior probability. In terms of the posterior probability, the PPV was 85.7% and the NPV 98.6%, indicating that this marker probably plays an equally significant role in both confirming and excluding the presence of the disease.

In conclusion, our findings revealed that plasma type IV collagen levels could be used as a novel, simple, and inexpensive indicator of nephropathy in T2DM. These levels hold promise as valuable biomarkers to predict progress of nephropathy, particularly during its early stages. The ACR positively correlates with type IV collagen. Consequently, measuring plasma IV collagen levels may allow earlier intervention to the complications of DN and the reduction of morbidity and mortality.

Author Contributions

FAA designed and approved the whole research protocol and amended the final paper version to be published. MMH contributed to the protocol design, revised laboratory work, monitored the data collection process, interpreted the data, provided clinical support, and revised the manuscript. HAE supervised sample collection according to inclusion criteria, revised clinical data, diagnosis, and patient classification. HSE collected the samples and clinical data, conducted the statistical analysis, and wrote the manuscript draft. All authors approved the manuscript.

Declaration of Conflicting Interests

The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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Ethical approval

The study protocol was reviewed and approved by the Research Ethics Committee of the Faculty of Medicine for Girls, Al-Azhar University (reference number: 2021101060).

Informed consent

Each study subject signed an informed consent letter before enrollment in the study..

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