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Recommended Citation

Hassan, Marwa E.; Mahfouz, Mohamed H.; and Shoman, Mohamed S. (2020) "Assessment of urinary type IV collagen, alpha-1 microglobulin, and transferrin in type 2 diabetes mellitus with nephropathy," *Journal of Medicine in Scientific Research*: Vol. 3: Iss. 1, Article 4.

DOI: https://doi.org/10.4103/JMISR.JMISR_74_19

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Assessment of urinary type IV collagen, alpha-1 microglobulin, and transferrin in type 2 diabetes mellitus with nephropathy

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Abstract

Aim

The aim of this study was to assess the levels of urinary type IV collagen, alpha-1 microglobulin, and transferrin as risk factors for early detection of nephropathy progression in diabetic patients.

Patients and methods

Four groups (25 patients each) participated in this study. Group I: controls, group II: type 2 diabetes mellitus (T2DM) with normoalbuminuria [albumin creatinine ratio (ACR)<30 mg/g], group III: T2DM with microalbuminuria, and group IV: T2DM with macroalbuminuria (ACR≥300 mg/g). Urinary type IV collagen, alpha-1 microglobulin, and transferrin levels were measured using enzyme-linked immunosorbent assay. The levels of glucose, glycosylated hemoglobin, lipids, urea, creatinine, and microalbumin were also measured.

Results

Significant elevation of glucose and glycosylated hemoglobin, total cholesterol, triglycerides as well as low-density lipoprotein cholesterol, and a significant reduction of high-density lipoprotein cholesterol were reported in diabetic nephropathy as compared with the control group and normoalbuminuric group ($P<0.05$). The dramatic significant increase in urinary type IV collagen, alpha-1 microglobulin, and transferrin levels were observed in diabetic patients with microalbuminuria and macroalbuminuria compared with those in the normoalbuminuric group ($P<0.05$). Type IV collagen, alpha-1 microglobulin, and transferrin were found to correlate positively and significantly with ACR in diabetic groups, and also type IV collagen and alpha-1 microglobulin were found to correlate positively and significantly with microalbumin, urea and creatinine in diabetic nephropathy groups.

Conclusions

Urinary type IV collagen, alpha-1 microglobulin, and transferrin levels are strongly associated with the prevalence of nephropathy in T2DM and could be promising useful biomarkers for predicting nephropathy progression, especially at early stages. Furthermore, they may serve as a tool to monitor the impact of prevention and intervention on renal damage.

Keywords: Alpha-1 microglobulin, transferrin, nephropathy, type 2 diabetes, type IV collagen

INTRODUCTION

Diabetes mellitus (DM) is an endocrine and metabolic disease that has a serious impact on human health. The morbidity and mortality of DM have risen continually at an alarming rate in recent years, and the population with DM is predicted to be about 552 million worldwide by 2030 [1]. The complications of DM include diabetic retinopathy, diabetic cardiovascular diseases, and diabetic nephropathy (DN), which is the most common and serious complication of DM [2].

DN occurs in approximately one-third of all people with diabetes and is the leading cause of renal failure. It is characterized by an increased urinary albumin excretion in the absence of other renal diseases. The earliest clinical evidence

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Access this article online

Quick Response Code:



Website:
www.jmsr.eg.net

DOI:
10.4103/JMISR.JMISR_74_19

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Submitted: 22-Nov-2019 Revised: 13-Jan-2020 Accepted: 29-Jan-2020 Published: 11-Jun-2020

How to cite this article: Mahfouz MH, Shoman MS, Hassan ME. Assessment of urinary type IV collagen, alpha-1 microglobulin, and transferrin in type 2 diabetes mellitus with nephropathy. J Med Sci Res 2020;3:18-27.

of nephropathy is the presence of low, but abnormal levels of albumin in the urine, which is known as microalbuminuria or incipient nephropathy [3].

Kidney Disease Improving Global Outcomes incorporates the albuminuria level given as albumin creatinine ratio (ACR) (mg/g creatinine) and divided into three albuminuria categories: normal-to-mildly increased albuminuria known as normoalbuminuria (ACR < 30 mg/g), moderately increased albuminuria previously known as microalbuminuria (ACR = 30–300 mg/g), and severely increased albuminuria, formerly known as macroalbuminuria (ACR > 300 mg/g) [4,5]. Progress to end-stage kidney disease is one of the common significant cause in the development of organ damage and indirectly increase mortality in diabetic patients [6].

DN pathogenesis is not clear but several mechanisms are believed to participate in its development such as hyperglycemia, advanced glycation end products (AGEs), protein kinase C, oxidative stress, inflammation, and poly (ADP-ribose) polymerase activation [6]. Therefore, we need to look for more sensitive and specific biomarkers with greater predictability that is earlier than microalbuminuria or those appearing at the same time [2].

Several glomerular and tubular biomarkers predicting the onset or progression of DN have been identified and are becoming increasingly important in clinical diagnostics. Urinary concentrations of these damage markers are elevated in diabetic patients and are associated with the severity of DN [7]. Tubular injury, as shown by the detection of tubular damage markers (alpha-1 microglobulin) in the urine, is a critical component of the early course of DN and has been suggested to contribute in a primary way, rather than a secondary manner, to the development of early DN [7]. Tubular biomarkers have shown that tubular dysfunction can be present early in DN, sometimes preceding glomerular injury [8].

Type IV collagen is a component of the glomerular basement membrane and of the mesangial matrix [9]. Urinary type IV collagen could reflect morphological renal alterations in patients with T2DM. A relationship between the severity of histological lesions and urinary type IV collagen was reported in patients with T2DM [10,11]. Type IV urinary collagen is considered to be a specific indicator of early DN [12–14].

Transferrin, a plasma protein, is very similar to albumin in weight. It is more readily filtered through the glomerular barrier than Albumin because it is less anionic. Urinary transferrin is considered to be a more sensitive marker of glomerular damage in diabetic patients based on theory analysis and experimental results [13]. Urinary transferrin excretion shows a good linear relationship with urinary albumin excretion in diabetic patients, and increased urinary transferrin excretion predicts the development of microalbuminuria in type 2 diabetic patients with normoalbuminuria [15].

Alpha-1 microglobulin is a small molecular weight protein (27 kDa) that is present in various body fluids. In healthy kidneys, it passes freely through the glomerular membranes, and about 99% is reabsorbed and catabolized by the proximal tubular cells [13]. Increased alpha-1 microglobulin in urine can therefore be an early sign of renal damage, primarily of the proximal tubules [7].

This study determined the role of urinary type IV collagen, alpha-1 macroglobulin, and transferrin in predicting the development and progression of early DN and deterioration of renal function.

PATIENTS AND METHODS

Patients

This study was conducted on 100 patients who were divided into four groups as follows: group I included 25 healthy normal participants matching the same age and socioeconomic status with diabetic patients. Group II included 25 patients of T2DM with normoalbuminuria (ACR < 30 mg/g creatinine). Group III included 25 patients of T2DM with microalbuminuria (ACR = 30–300 mg/g creatinine). Group IV included 25 patients of T2DM with macroalbuminuria (ACR > 300 mg/g creatinine). The last three diabetic groups were classified according to urinary albumin excretion.

Patients with T2DM were selected from patients who regularly visited the outpatient clinic of the National Institute for Diabetes and Endocrinology. Type 2 diabetic patients were diagnosed in accordance with the criteria of the WHO [16]. Patients with a previous history of chronic disease of the kidneys, pancreas, or liver, with other known existing disease and; active inflammatory diseases were excluded. This study was approved by the research ethics committee of General Organization of Teaching Hospitals and Institutes. Written informed consent was obtained from controls and all patients.

After 12 h of overnight fasting, venous blood samples were collected from controls and diabetic patients into three types of vacutainer tubes and were processed as follows: first vacutainer tube with ethylenediaminetetraacetic acid (lavender cap) without centrifugation (whole blood sample) for assaying glycosylated hemoglobin (HbA1c); second tube with sodium fluoride (gray cap) for assaying of plasma glucose at once. The third tube without additives (red cap) in which blood was centrifuged at 4000 rpm for 10 min Sera were rapidly separated for assaying of lipid profile [cholesterol, triacylglycerol, high-density lipoprotein cholesterol (HDL-C), and low-density lipoprotein cholesterol (LDL-C)], urea, and creatinine. Hemolyzed samples were excluded. Fresh early morning urine sample was Collected from each subject into a sterile container and is used for determination of type IV collagen, alpha-1 microglobulin, transferrin, microalbumin, and ACR.

Methods

Quantitative determination of glucose was carried out colorimetrically using a commercial kit purchased

from Randox Laboratories (crumlin, UK) following the method of Takeda *et al.* [17]. Quantitative estimation of serum cholesterol was done colorimetrically using a commercial kit purchased from Randox Laboratories using the method of Richmond [18]. Serum HDL-C was assayed colorimetrically using a commercial kit purchased from Randox Laboratories using the method of Assmann [19]. LDL-C was quantified in serum using a kit provided by Spinreact for the quantitative determination of serum LDL-C following the method of Okada *et al.* [20]. Triglycerides (TG) in serum were measured colorimetrically using a commercial kit purchased from Randox Laboratories according to the method of Jacobs and Van Denmark [21]. Glycated hemoglobin was measured By ion exchange high performance liquid chromatography according to the method of Nathan *et al.* [22]. Serum urea concentration was quantitatively measured according to Tiffany *et al.* [23]. Colorimetrically, the amount of creatinine in serum and urine was quantitatively measured according to Vasiliades [24].

Microalbumin assay (turbidimetric immunoassay) Was used for the quantitative measurement of albumin in human urine on the ARCHITECT c8000TM System. The kit was provided by Abbott Diagnostics (ILLIONIS, UA). It is Important to compare the amount of albumin in the urine sample against its concentration of creatinine. This is termed the ACR expressed in ‘mg/g creatinine.’ The use of ACR for these random samples might replace 24-h urine collections; thus, ACR may be a useful diagnostic tool than microalbumin itself [25]. ACR was estimated by dividing the microalbumin concentration (mg/l) over the urine creatinine concentration (g/l). ACR was thus expressed in mg/g creatinine. Urinary type IV collagen concentration was measured using the enzyme-linked immunosorbent assay (ELISA) method. The kit was provided by ELISA kit no. ABIN414948 [26]. Alpha-1 microglobulin concentration was measured by using the sandwich ELISA method. The kit was provided by ELISA kit no. ab108884 [27]. Urinary transferrin was determined by an assay that uses the quantitative sandwich enzyme immunoassay technique. The kit was provided by ELISA kit no. ab108902 [28].

Statistical analysis

Data were statistically expressed as mean \pm SD. Comparison of more than two variables was analyzed by one-way analysis of variance followed by the Tukey–Kramer multiple comparison test. Correlations between various variables were calculated using the Pearson correlation coefficient. A *P* value of less than 0.05 was considered statistically significant. All statistical calculations were done using Statistical Package for the Social Sciences, version 20 (SPSS Inc., Chicago, Illinois, USA). Receiver-operating characteristic (ROC) curves were utilized to assess the diagnostic performance of type IV collagen, alpha-1 microglobulin, and transferrin.

RESULTS

Table 1 illustrated analysis of variance (*P* value) for demographic and biochemical characteristics of control and diabetic patients. It showed that no statistically significant differences were observed in terms of age and BMI between the studied groups. However, there was a statistically significant difference in the duration of diabetes among the microalbuminuric and macroalbuminuric diabetic groups (groups III and IV) as compared with group II. Significant higher systolic and diastolic blood pressure levels were observed in microalbuminuric and macroalbuminuric diabetic groups (groups III and IV) as compared with the control group and normoalbuminuric groups (groups I and II). The macroalbuminuric diabetic group (group IV) also showed a significant difference in systolic and diastolic blood pressure levels as compared with the microalbuminuric diabetic group (group III; *P* < 0.05). The mean levels of fasting plasma glucose (FPG) and HbA1c% were significantly higher (*P* < 0.05) in normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups (groups II, III, and IV) as compared with the control group (group I), and also a significant difference was observed in microalbuminuric and macroalbuminuric diabetic groups compared with the normoalbuminuric group (group II).

The results in Table 2 showed that there was no significant difference in TG between the macroalbuminuric diabetic group (group IV) and the normoalbuminuric group (group II) and between microalbuminuric, and macroalbuminuric diabetic groups (groups III and IV). There was a statistically significant difference in cholesterol, TG, HDL, and LDL among the normoalbuminuric, microalbuminuric and macroalbuminuric diabetic groups as compared with the control group. Moreover, the macroalbuminuric diabetic group (group IV) showed a significant difference in HDL and LDL as compared with the microalbuminuric diabetic group (group III). A significant difference in cholesterol, HDL, and LDL were observed in macroalbuminuric diabetic groups (group IV) as compared with the normoalbuminuric group (group II).

The results in Table 3 showed that serum urea and creatinine were significantly increased in diabetic patients with microalbuminuric and macroalbuminuric diabetic groups (groups III and IV) as compared with normal controls (group I) and significantly increase in the macroalbuminuric diabetic group (group IV) as compared with the normoalbuminuric diabetic group (group II). Urinary microalbumin and ACR showed a significant change in microalbuminuric and macroalbuminuric diabetic groups (groups III and IV) with the control group (group I). In addition, urinary microalbumin and ACR were markedly increased in microalbuminuric and macroalbuminuric diabetic groups compared with the normoalbuminuric diabetic group (group II). Urinary microalbumin, ACR, and creatinine were significantly increased in diabetic patients with macroalbuminuria compared with the microalbuminuric diabetic group (group III).

The results in Table 4 showed that the urinary levels of type IV collagen, alpha-1 microglobulin and transferrin were significantly increased in normoalbuminuric, microalbuminuric and macroalbuminuric diabetic groups (groups II, III, and IV) compared with the control group (group I) and also in microalbuminuric and macroalbuminuric diabetic groups (groups III and IV) compared with the normoalbuminuric group (groups II). A significant difference was observed in the macroalbuminuric diabetic group compared with the microalbuminuric group.

Table 5 illustrated significant correlations between type IV collagen and other biochemical parameters in nephropathy groups. It showed a significant correlation between TG, creatinine, transferrin, and ACR and type IV collagen in the normoalbuminuric diabetic group (groups II), and between

ACR and type IV collagen in the microalbuminuric diabetic group (group III). In addition, significant correlation was observed between urea, microalbumin, and type IV collagen in the macroalbuminuric diabetic group (group IV).

Table 6 illustrated significant correlations between transferrin and other biochemical parameters in nephropathy groups. It showed a significant correlation between fasting blood glucose (FBG), HDL, ACR, and transferrin in the normoalbuminuric diabetic group (groups II). In addition, significant correlation observed between FBG, HDL, and transferrin in the macroalbuminuric diabetic group (group IV).

Table 7 Illustrated significant correlations between alpha-1 microglobulin and other biochemical parameters in nephropathy groups. It showed a significant correlation between creatinine,

Table 1: Demographic and biochemical characteristics of control and diabetic patients (mean±SD)

Parameters	Group I	Group II	Group III	Group IV
Age (years)	45.0±2.6	53.0±2.90	52.0±2.4	56.0±3.5
Sex (F/M)	13/12	14/11	11/14	15/10
Duration of diabetes (years)	-	4.3±0.27	9.3±1.25 ^b	9.8±1.30 ^b
BMI (kg/m ²)	25.10±0.5	26.81±0.35	27.10±0.45	27.18±0.70
SBP (mmHg)	119.6±7.7	125.4±10.5	140.5±4.8 ^{a,b}	160.1±3.5 ^{a,b,c}
DBP (mmHg)	70.6±3.9	80.0±6.5	90.3±4.7 ^{a,b}	100.9±6.1 ^{a,b,c}
FBG (mg/dl)	88.48±7.42	233.56±68.45 ^a	285.68±66.45 ^{a,b}	291.88±67.91 ^{a,b}
HbA1c (%)	5.36±0.53	8.20±0.74 ^a	9.63±1.32 ^{a,b}	9.74±1.47 ^{a,b}

DBP, diastolic blood pressure; F, female; FBG, fasting blood glucose; HbA1c, glycosylated hemoglobin; M, male; SBP, systolic blood pressure. ^aP value less than 0.05 versus group I. ^bP value less than 0.05 versus group II. ^cP value less than 0.05 versus group III.

Table 2: Lipid profile in the different studied groups (mean±SD)

Parameters	Group I	Group II	Group III	Group IV
T-cholesterol (mg/dl)	174.20±19.30	227.48±40.63 ^a	244.48±44.73 ^a	256.92±56.28 ^{a,b}
HDL-C (mg/dl)	42.40±7.21	36.32±8.95 ^a	37.28±7.47 ^a	26.04±7.98 ^{a,b,c}
LDL-C (mg/dl)	113.1±15.39	163.20±20.82 ^a	168.48±25.30 ^a	211.56±31.85 ^{a,b,c}
TG (mg/dl)	108.16±20.45	190.40±46.44 ^a	193.28±45.86 ^a	196.84±48.79 ^a

HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; T-cholesterol, total cholesterol; TG, triglycerides. ^aP value less than 0.05 versus group I. ^bP value less than 0.05 versus group II. ^cP value less than 0.05 versus group III.

Table 3: Kidney function, microalbumin, and albumin creatinine ratio levels in the different studied groups (mean±SD)

Parameters	Group I	Group II	Group III	Group IV
Urea (mg/dl)	23.52±4.19	24.48±4.51	26.60±5.44 ^a	29.20±5.84 ^{a,b}
Creatinine (mg/dl)	0.84±0.16	0.87±0.15	0.93±0.16 ^a	1.13±0.27 ^{a,b,c}
Microalbumin (mg/ml)	7.25±3.12	7.62±3.13	57.32±13.21 ^{a,b}	99.36±11.45 ^{a,b,c}
ACR (mg/g)	14.69±5.875	18.04±6.53	135.00±25.09 ^{a,b}	370.88±51.65 ^{a,b,c}

ACR, albumin creatinine ratio. ^aP value less than 0.05 versus group I. ^bP value less than 0.05 versus group II. ^cP value less than 0.05 versus group III.

Table 4: Urinary level of type IV collagen, alpha-1 microglobulin, and transferrin in the different studied groups (mean±SD)

Parameters	Group I	Group II	Group III	Group IV
Type IV collagen (ng/mg)	1.01±0.19	1.88±0.34 ^a	4.56±1.41 ^{a,b}	12.67±1.96 ^{a,b,c}
Alpha-1 microglobulin (mg/g creatinine)	3.25±1.10	10.34±2.59 ^a	19.92±4.52 ^{a,b}	39.84±4.98 ^{a,b,c}
Transferrin (mg/g creatinine)	1.39±0.53	2.15±0.39 ^a	20.08±5.79 ^{a,b}	62.04±17.44 ^{a,b,c}

^aP value less than 0.05 versus group I. ^bP value less than 0.05 versus group II. ^cP value less than 0.05 versus group III.

Table 5: Significant correlations between type IV collagen and other biochemical parameters in nephropathy groups

Parameters	Group II type IV collagen (ng/mg)		Group III type IV collagen (ng/mg)		Group IV type IV collagen (ng/mg)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
ACR (mg/g)	0.478	0.016	0.3729	0.05	-	-
Creatinine (mg/dl)	0.356	0.080	-	-	-	-
TG (mg/dl)	0.4816	0.015	-	-	-	-
Transferrin (mg/g creatinine)	0.5655	0.003	-	-	-	-
Urea (mg/dl)	-	-	-	-	0.5145	0.008
Microalbumin (mg/ml)	-	-	-	-	0.474	0.017

r, Pearson's correlation coefficient; TG, triglycerides.

Table 6: Significant correlations between transferrin and other biochemical parameters in nephropathy groups

Parameters	Group II transferrin (mg/g creatinine)		Group IV transferrin (mg/g creatinine)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
ACR (mg/g)	0.4712	0.018	-	-
FBG (mg/dl)	0.4302	0.032	0.4553	0.022
HDL-C (mg/dl)	-0.4585	0.021	0.4500	0.024

FBG, fasting blood glucose; HDL-C, high-density lipoprotein cholesterol; *r*, Pearson's correlation coefficient.

cholesterol, type IV collagen, ACR, and alpha-1 microglobulin in normoalbuminuric diabetic groups (groups II), and between urea, creatinine, TG, microalbumin, and alpha-1 microglobulin in the microalbuminuric diabetic group (group III). In addition, significant correlation was observed between urea, creatinine, type IV collagen, microalbumin, and alpha-1 microglobulin in the macroalbuminuric diabetic group (group IV).

The ROC curve was designed for type IV collagen, alpha-1 microglobulin, and transferrin (Table 8, Fig. 1) to discriminate normoalbuminuric diabetic patients from microalbuminuric diabetic patients. The cutoff values for type IV collagen, transferrin, and alpha-1 microglobulin were 2.6 ng/mg, 2.9 mg/g creatinine, and 10.2 mg/g creatinine, respectively. Area under the curve for type IV collagen, transferrin, and alpha-1 microglobulin was 0.916, 0.850, and 0.767, respectively. This result indicates the good validity of the above markers particularly type IV collagen to differentiate normoalbuminuric diabetic patients from microalbuminuric diabetic patients.

The ROC curve was designed for type IV collagen, alpha-1 microglobulin, and transferrin (Table 9, Fig. 2) to discriminate normoalbuminuric diabetic patients from macroalbuminuric diabetic patients. The cutoff values for type IV collagen, transferrin, and alpha-1 microglobulin were 2.7 ng/mg, 2.7 mg/g creatinine, and 10.3 mg/g creatinine, respectively. Area under the curve for type IV collagen, transferrin, and alpha-1 microglobulin was 0.964, 0.858, and 0.772, respectively. This result indicates the good validity of the above markers particularly type IV collagen to differentiate normoalbuminuric diabetic patients from macroalbuminuric diabetic patients.

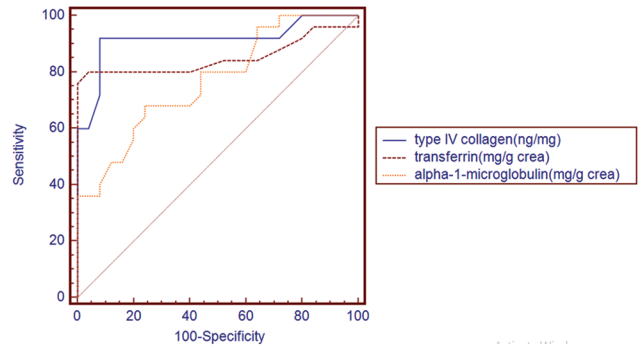


Figure 1: ROC curves for differentiation between normoalbuminuric diabetic patients and microalbuminuric diabetic patients by type IV collagen, α -1 microglobulin, and transferrin ($P=0.0001$).

DISCUSSION

DN is an important cause of chronic kidney disease which often leads to end-stage renal disease. DN is one of the major complications in diabetic patients. DN occurs in ~ 20–40% of patients with T2DM [29].

Early and accurate identification of DN is important to improve clinical outcomes. Clinically, the appearance of pathological albuminuria, microalbuminuria, is considered a marker of early onset of DN. However, a substantial proportion of renal impairment occurs among normoalbuminuric diabetic patients and is associated with more advanced diabetic glomerular lesions and increased risk of progression [30–32]. The main risk factors associated with the development of DN include dyslipidemia, poor glycemic control, smoking, and hypertension [33].

Several glomerular and tubular biomarkers predicting the onset or progression of DN have been identified and they are important in the clinical diagnosis of DN. Urinary concentrations of these damage markers (both glomerular and tubular) are increased in diabetic patients and are associated with the severity of DN [34]. Therefore, more sensitive and specific markers such as type IV collagen, alpha-1 microglobulin, and transferrin in addition to microalbuminuria were used to predict the development and progression of DN in patients with T2DM even at the very early stage of the disease (normoalbuminuric DN).

Table 7: Significant correlations between alpha-1 microglobulin and other biochemical parameters in nephropathy groups

Parameters	Group II alpha-1 microglobulin (mg/g creatinine)		Group III alpha-1 microglobulin (mg/g creatinine)		Group IV alpha-1 microglobulin (mg/g creatinine)	
	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>	<i>r</i>	<i>P</i>
Type IV collagen (ng/mg)	0.431	0.032	-	-	0.453	0.023
ACR (mg/g)	0.499	0.011	-	-	-	-
Creatinine (mg/dl)	0.500	0.011	0.436	0.029	0.424	0.035
T-cholesterol (mg/dl)	0.384	0.051	-	-	-	-
Urea (mg/dl)	-	-	0.556	0.004	0.485	0.014
Microalbumin (mg/ml)	-	-	0.510	0.009	0.438	0.029
TG (mg/dl)	-	-	0.412	0.041	-	-

r, Pearson's correlation coefficient; TG, triglycerides.

Table 8: Comparison between characteristic receiver-operating characteristic curves for type IV collagen, alpha-1 microglobulin, and transferrin in normoalbuminuric diabetic patients and microalbuminuric diabetic patients

Variables	AUC	Cut off	Sensitivity (%)	Specificity (%)	<i>P</i>
Type IV collagen (ng/mg)	0.916	2.6	92	92	0.0001
Transferrin (mg/g creatinine)	0.850	2.9	80	96	0.0001
Alpha-1 microglobulin (mg/g creatinine)	0.767	10.2	68	76	0.0001

AUC, area under the curve.

Table 9: Comparison between characteristic receiver-operating characteristic curves for type IV collagen, alpha-1 microglobulin, and transferrin in normoalbuminuric diabetic patients and macroalbuminuric diabetic patients

Variables	AUC	Cut off	Sensitivity (%)	Specificity (%)	<i>P</i>
Type IV collagen (ng/mg)	0.964	2.7	96	92	0.0001
Transferrin (mg/g creatinine)	0.858	2.7	84	84	0.0001
Alpha-1 microglobulin (mg/g creatinine)	0.772	10.3	72	72	0.0001

AUC, area under the curve.

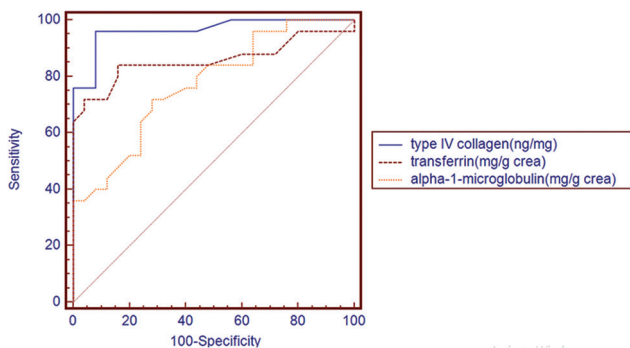


Figure 2: Curves for differentiation between normoalbuminuric diabetic patients and macroalbuminuric diabetic patients by type IV collagen, α -1 microglobulin, and transferrin ($P=0.0001$).

The results obtained in this study showed that no statistically significant differences were observed in terms of age and BMI between the studied groups. However, there was a statistically significant difference in the duration of diabetes among the microalbuminuric and macroalbuminuric diabetic groups as compared with the normoalbuminuric group. Significant higher systolic and diastolic blood pressure levels were observed in microalbuminuric and macroalbuminuric diabetic groups as

compared with the control group and the normoalbuminuric group. The macroalbuminuric diabetic group also showed a significant difference in systolic and diastolic blood pressure levels as compared with the microalbuminuric diabetic group ($P < 0.05$). The mean levels of FPG and HbA1c% were significantly higher ($P < 0.05$) in normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups as compared with the control group. Significant difference was observed in microalbuminuric and macroalbuminuric diabetic groups compared with the normoalbuminuric group. These results are in conformity with those of Mahendran *et al.* [10], who stated that the duration of diabetes and systolic blood pressure was significantly increased in diabetic patients when compared with healthy controls. In addition, the levels of FPG and HbA1c% were significantly increased in normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups as compared with the healthy group and in macroalbuminuric diabetic groups when compared with microalbuminuric, and macroalbuminuric diabetic groups. The studies of Wu *et al.*[35] and Miedema[36] have shown that the elevated blood glucose level leads to the elevated attachment of glucose molecules to the hemoglobin in the erythrocyte and this leads to an increase in HbA1c. In DM, higher amounts of

HbA1c, indicate a poorer control of blood glucose levels with consequent complications such as nephropathy, cardiovascular disease, retinopathy, and neuropathy.

This study found that there was no significant difference in TG between the macroalbuminuric diabetic group and the normoalbuminuric group and between microalbuminuric, and macroalbuminuric diabetic groups. There was a statistically significant difference in cholesterol, TG, HDL, and LDL among the normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups as compared with the control group. Moreover, the macroalbuminuric diabetic group showed a significant difference in HDL and LDL as compared with the microalbuminuric diabetic group. A significant difference in cholesterol, HDL, and LDL were observed in macroalbuminuric diabetic groups as compared with the normoalbuminuric group. The study of Zhang *et al.*[37] showed that the levels of cholesterol and TG were significantly high in the macroalbuminuric diabetic group than the normoalbuminuric group and the healthy group. In addition Mahendran *et al.*[10] found a significant difference in TG between the macroalbuminuric diabetic group and the normoalbuminuric group and the healthy group and also between microalbuminuric and macroalbuminuric diabetic groups. There was a statistically significant difference in cholesterol, HDL, and LDL among the normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups as compared with the control group. Also, he showed a significant difference in LDL and cholesterol levels between the macroalbuminuric diabetic group and the normoalbuminuric group. These results were explained by Krauss[38] who explained that insulin resistance may contribute to the development of dyslipidemia in diabetic patients where insulin resistance increases the flow of free fatty acids from the adipose tissue and leads to impairment of uptake of free fatty acids in the skeletal muscle leading to increased fatty acid flow to the liver [39,40]. An increase in free fatty acid levels has been found in individuals with impaired glucose tolerance suggesting that insulin resistance is associated with an increased level of free fatty acid which occurs before the onset of hyperglycemia [41]. Some studies have shown that insulin resistance elevates the activity of hepatic lipase, which is responsible for hydrolysis of phospholipids into HDL and LDL particles with consequent formation of very small and dense LDL particles and a reduction in HDL particles [42,43].

This study showed that serum urea and creatinine were significantly increased in microalbuminuric and macroalbuminuric diabetic groups as compared with the normal control and significantly increased in the macroalbuminuric diabetic group as compared with the normoalbuminuric diabetic group. Urinary microalbumin and ACR showed a significant change in microalbuminuric and macroalbuminuric diabetic groups compared with the control group. In addition, urinary microalbumin and ACR were markedly increased in microalbuminuric and macroalbuminuric diabetic groups compared with the normoalbuminuric diabetic group. Urinary

microalbumin, ACR, and creatinine were significantly increased in diabetic patients with macroalbuminuria compared with the microalbuminuric diabetic group. These findings are in agreement with Mahfouz *et al.*[44] who found that serum urea and, creatinine were significantly elevated in diabetic patients with Microalbuminuria and macroalbuminuria as compared with healthy participants and there was a pronounced increase in the macroalbuminuric diabetic group as compared with the microalbuminuric group. Also, in diabetic patients with microalbuminuria and macroalbuminuria, urinary microalbumin was markedly elevated compared with healthy participants. Urinary ACR was significantly increased in diabetic patients with microalbuminuria and macroalbuminuria compared with healthy participants and Normoalbuminuric diabetic patients. On the other hand, urinary Macroalbuminuric and ACR were significantly elevated in macroalbuminuric diabetic patients than in the microalbuminuric diabetic patients. Several hypotheses explained the relationship between microalbuminuria and DN, where they suggested that vascular endothelium dysfunction causes both microalbuminuria and DN [45–47]. Endothelial dysfunction can be defined as any change in the properties of endothelium that are unsuitable to the preservation of organ function. Therefore, many types of dysfunction of the endothelium could exist depending on which function is affected (e.g. the regulation of vasomotor activity, fibrinolysis and hemostasis, permeability of macromolecules, vascular smooth muscle cell proliferation, and leukocyte adhesion). In DN, hyperglycemia produces AGEs within the plasma and tissues [45,46]. These are produced via the nonenzymatic oxidation reaction of amino acids from proteins that exist in the plasma and renal tissue [45,46]. AGEs induce renal complications by two pathways. One by remaining irreversibly bound to the tissue protein such as matrix proteins (type IV collagen) and impairs their degradation by matrix metalloproteinases which contribute to fibrosis via excess accumulation of extracellular matrix proteins [48–50]. Second, by interacting with the receptor for AGE expressed by podocytes and endothelial and mesangial cells in the kidneys, causing hyperglycemia and generating AGEs which lead to basement membrane thickening, glomerular extracellular matrix accumulation, the effacement of podocytes and tubulointerstitial fibrosis. These factors initiate pathological changes via the activation of a cascade of mediators at different stages during the systematic progression of the disease, such as high molecular weight proteins (albumin), cytokines, growth factors and exosomes [51]. Therefore, the association of microalbuminuria with generalized endothelial dysfunction could explain why microalbuminuria strongly predicts DN [51].

This study showed that the urinary level of type IV collagen, alpha-1 microglobulin, and transferrin were significantly increased in normoalbuminuric, microalbuminuric, and macroalbuminuric diabetic groups compared with the control group and also in microalbuminuric and macroalbuminuric diabetic groups compared with the normoalbuminuric groups.

Significant difference was observed in the macroalbuminuric diabetic group compared with the microalbuminuric group. This is in accordance with the results obtained by Mahendran *et al.*[10] who observed that the levels of urinary type IV collagen were significantly elevated in type 2 diabetic patients compared with healthy controls and there was also a significant difference observed in macroalbuminuric and microalbuminuric diabetic patients compared with normoalbuminuric diabetic patients. High glucose level stimulates type IV collagen production by activating the cellular transforming growth factor beta (TGF- β) system [52]. Specifically, high glucose increases the secretion of endogenous TGF- β 1 that then acts upon the cell in an autocrine manner to activate the expression of collagen IV and other extracellular matrix proteins [53]. Hyperglycemia stimulates various inflammatory pathways both directly and via gene transcription factors which stimulate oxidative stress, renin-angiotensin system, TGF- β , and monocyte chemoattractant protein-1. This leads to malfunction, podocyte injury, deposition of proteins in the extracellular matrix of the nephron with albumin leak and apoptosis [54,55]

Matheson *et al.*[56] have reported that urinary excretion of transferrin was higher in Microalbuminuric diabetic patients than in normoalbuminuric diabetic patients. Jiang *et al.*[7] explained that transferrin is a protein very similar in weight to albumin, but slightly larger. It is less anionic than albumin with an isoelectric point one unit higher; therefore, it can filter more readily through the glomerular barrier. Urinary transferrin excretion has also been correlated with the degree of tubular atrophy, interstitial fibrosis, and interstitial inflammatory cell infiltration [57]. Transferrin excretion is higher in diabetic patients compared with healthy controls, even before they develop microalbuminuria [58,59]. Because diabetic patients are more likely to have transferrinuria than albuminuria [60,61], and because the albumin/transferrin ratio was significantly smaller in normoalbuminuric and microalbuminuric diabetic patients compared with macroalbuminuric diabetic patients, urinary transferrin is considered to be a more sensitive marker of glomerular damage in diabetic patients [60,62]. Furthermore, increased urinary transferrin excretion predicts the development of microalbuminuria in type 2 diabetic patients with normoalbuminuria [63,64].

Zhang *et al.*[65] found that levels of urinary alpha-1 microglobulin in the DN groups were significantly higher than those of the diabetes group and control, suggesting statistically significant differences ($P < 0.05$) and this is because of its low molecular weight. The free form of alpha-1 microglobulin is filtered freely through the renal glomerular basement membrane and reabsorbed by the proximal tubular cells [66]. Hence, any proximal tubular cell dysfunction results in increased quantities of alpha-1 microglobulin in urine. The level of urinary alpha-1 microglobulin was found to be elevated in both type 1 and type 2 diabetic patients. In type 2 diabetic patients, alpha-1 microglobulin excretion was directly

correlated with HbA1c and albuminuria, and was decreased with improved glycemic control in Caucasians [67,68]. Similar findings have been shown in Asian population [69].

This study found significant correlations between type IV collagen and other biochemical parameters in nephropathy groups. It showed that there was significant correlation between TG, creatinine, transferrin, and ACR and type IV collagen in the normoalbuminuric diabetic group, and between ACR and type IV collagen in the microalbuminuric diabetic group. In addition, significant correlation was observed between urea, microalbumin, and type IV collagen in the macroalbuminuric diabetic group. This is in accordance with the results obtained by Mahendran *et al.* [10], who observed positive and significant correlation of ACR with type IV collagen. Also Banu *et al.*[70] observed that there was no correlation between urinary IV-collagen levels and fasting serum cholesterol level, but there was a significant correlation between serum IV-collagen and TG levels and creatinine.

High levels of glucose stimulate type IV collagen synthesis and may reduce its breakdown by producing advanced glycosylation of proteins. As a consequence, increased deposition of type IV collagen has been observed in the glomerular mesangial matrix of diabetic kidneys with diffuse glomerulosclerosis [12,13]. Additionally, urinary type IV collagen excretion has been associated with mesangial expansion and tubulointerstitial and glomerular injury [11,14].

This study found significant correlations between transferrin and other biochemical parameters in nephropathy groups. It showed that there was significant correlation between FBG, HDL, ACR, and transferrin in the normoalbuminuric diabetic group. In addition, significant correlation was observed between FBG, HDL, and transferrin in the macroalbuminuric diabetic group. These findings are in contrast to the results of Jiang *et al.* [7], who found that urinary transferrin excretion is not correlated with glycemic control (HbA1c and fasting glucose), supporting the hypothesis that transferrinuria is caused by intrinsic renal damage.

Our study found significant correlations between alpha-1 microglobulin and other biochemical parameters in nephropathy groups. It showed that there was significant correlation between creatinine, cholesterol, type IV collagen, ACR, and alpha-1 microglobulin in the normoalbuminuric diabetic group and between urea, creatinine, TG, microalbumin, and alpha-1 microglobulin in the microalbuminuric diabetic group. In addition, significant correlation observed between urea, creatinine, Type IV collagen, microalbumin, and alpha-1 microglobulin in the macroalbuminuric diabetic group. These findings are in agreement with Hong *et al.* [69], who found that the level of alpha-1 microglobulin significantly increased with the severity of albuminuria. Also, Saif and Soliman[71] found that the excretion of alpha-1 microglobulin was directly correlated with albuminuria and HbA1c levels, and it was decreased with improved glycemic control.

Our sensitivity and specificity analysis of urinary IV collagen, alpha-1 microglobulin, and transferrin excretion showed that IV collagen could be a more sensitive indicator of early nephropathy in DM than alpha-1 microglobulin and transferrin.

CONCLUSION

In conclusion, urinary IV collagen, alpha-1 microglobulin, and transferrin levels are strongly associated with the prevalence of nephropathy in T2DM and could be promising useful biomarkers for predicting nephropathy progression, especially in early stages. Furthermore, they may serve as a tool to monitor the impact of prevention and intervention on renal damage.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

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