



## Urinary Type IV Collagen Levels in Syrian Type II Diabetic Nephropathy Patients

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### ABSTRACT

This research aimed to study and assess the diagnostic value of urinary type IV collagen levels in type II diabetic nephropathy patients. We measured urinary collagen IV levels by enzyme immunoassay (EIA) in 25 normal subjects and in 139 type II diabetic patients, with various degrees of urinary albumin excretion rate: (normoalbuminuric, microalbuminuric and macroalbuminuric) and studied its relationship to renal markers (albumin:creatinine (A:C) ratio - and estimated creatinine clearance eCcr) and glycemia control marker (HbA1). Urinary collagen IV levels were significantly higher in all 3 groups of patient's more than normal subjects and significantly higher in microalbuminuric and macroalbuminuric groups more than normoalbuminuric group. Urinary collagen IV levels correlate positively with A:C ratio, inversely with eCcr (more significantly than correlation of A:C ratio with eCcr in normoalbuminuric and microalbuminuric patients) and positively with HbA1 in microalbuminuric and macroalbuminuric groups, but not in normoalbuminuric patients. It may be considered that urinary type IV collagen as noninvasive and early marker for diabetic nephropathy before microalbuminuria stage or declining of filtration function.

**Keywords:** Collagen IV, type II diabetic nephropathy, albumin.

### INTRODUCTION

DN is classically understood as a glomerular disease. Five different stages are recognized, beginning with glomerular hyperfiltration, and progressing through incipient nephropathy, micro albuminuria, overt proteinuria, finally to ESRD (end-stage renal disease). Persistent micro albuminuria is considered as incipient DN. Overtime, micro albuminuria may progress to overt (dipstick positive) proteinuria, which is the hallmark of established DN.<sup>1</sup>

Diabetic nephropathy seems to occur as a result of an interaction between metabolic and hemodynamic factors.

Glucose dependent pathway is activated with in diabetic kidney. These include increased oxidative stress and accumulation of advanced glycated end-product.

Hemodynamic factors are also implicated in the pathogenesis of diabetic nephropathy and include increased systemic and intraglomerular pressure and activation of various vasoactive hormone pathways including rennin-angiotensin and endothelin.<sup>2</sup>

Diabetic nephropathy refers to a characteristic set of structural and functional kidney abnormalities that occur in patients with diabetes. Although best described in patients with type I diabetes, similar findings are now known to occur in the more common type II diabetic patient. Structural abnormalities include hypertrophy of the kidney, an increase in the thickness of glomerular basement membranes, accumulation of extracellular matrix (ECM) components in the glomerulus (nodular and diffuse glomerulosclerosis), tubular atrophy, and interstitial fibrosis. Functional alterations include an early

increase in the glomerular filtration rate with intraglomerular hypertension, subsequent proteinuria, systemic hypertension, and eventual loss of renal function.<sup>3</sup>

Type IV collagen, fibronectin and laminin, which are normal constituents of the mesangium and glomerular basement membrane (GBM) are increased in diabetic kidney disease.<sup>4</sup>

In order to assess accurately the morphological changes in the target organs and the extent to which the fibrotic changes affect their function, invasive studies (tissue biopsy) is commonly required. However, biopsy is not always feasible in human tissues and is associated with obvious risks. Therefore it is highly desirable to potentially estimate the severity of organ fibrosis by measuring ECM factors in biological fluids (peripheral blood or urine). Such markers, if proven they mirror the changes in specific organs structure and function, will allow a better monitoring of the disease. Moreover, if these markers levels show changes with treatment, they will be a useful tool to evaluate the therapeutic interventions. Thus the potential clinical value of the circulating or urinary levels of several ECM components and its regulators have been tested in diabetes and its complications.<sup>4</sup>

Type IV collagen in the circulation or urine has been identified as a possible indicator of renal injuries, especially in early stages of diabetic nephropathy, in numerous, albeit relatively small studies. Both serum and urinary type IV collagen increased in accordance with the clinical stage of the renal disease.<sup>4</sup>



Because of the importance of these markers, we have studied urinary collagen type IV levels and asses their diagnostic value in type II diabetic nephropathy Syrian patients as collagen type IV is a normal component of renal mesangium and glomerular basement membrane (GBM).

### SUBJECTS, MATERIALS AND METHODS

The study group consisted of 25 healthy volunteers and 139 subjects with type 2 diabetes who had a wide range of duration of diabetes (5 months –35 years). All patients were currently being treated and were receiving insulin, oral hypoglycemic agents, or both except one patient who was being treated with a diabetic diet. About of 55% of patients had been taking hypertension medication such as: ACE inhibitors, angiotensin receptor antagonists,  $\beta$ -blockers,  $Ca^{2+}$  channel blockers and diuretics. Measurement of HbA1, fasting glucose and serum creatinine levels was performed. Urine samples for measurement of albumin, creatinine, and type IV collagen also were obtained in each subject. Estimated creatinine clearance rate (eCCr) was calculated from Cockcroft-Gault formula, which says:

$$eC_{Cr} = \frac{(140 - \text{Age}) \times \text{Mass (in kilograms)} \times [0.85 \text{ if Female}]}{72 \times \text{Serum Creatinine (in mg/dL)}}$$

The eCcr value was in ml/min.

Where the serum creatinine value (in milligrams per deciliter), the mass (in kilograms) and the age were taken.<sup>5</sup>

Because the purpose of the study was to assess diagnostic value of the urinary collagen IV in diabetic nephropathy, serum creatinine between 0.5 and 1.7 mg/dl was required for inclusion.

We excluded gestational diabetic women, diabetic patients with ESRD stage, type I diabetic patients and diabetic patients with non diabetic renal diseases.

#### Measurement of HbA1 levels

Glycohemoglobin HbA1 levels were measured by fast ion-exchange resin separation method (Human Gesellschaft für Biochemica und Diagnostica mbH, Germany).

#### Measurement of fasting glucose levels

Serum fasting glucose levels (mg/dl) were measured by enzymatic colorimetric assay (Roche Diagnostics, Germany) in Hitachi 912 analyzer.

#### Measurement of serum/ urine creatinine level

Serum/urine creatinine levels (mg/dl) were measured by kinetic colorimetric assay - Jaffé method- from (Roche Diagnostics, Germany) in Hitachi 912 analyzer.

#### Measurement of urine albumin levels

Urine albumin levels (mg/dl) in- morning urine samples- were measured by immunoturbidimetric assay (Roche Diagnostics, Germany) in Hitachi 912 analyzer.

#### Calculation of urinary albumin: creatinine (A:C) ratio (mg / g)

Urinary A: C ratio (mg/g) =urinary albumin (mg/dl) / urinary creatinine (g/dl).

#### Measurement of urinary type IV Collagen levels

Urinary type IV Collagen levels ( $\mu\text{g/L}$ ) in- morning urine samples- were measured by enzyme immunoassay (Argutus Medical, Ireland). It is a solid phase one-step sandwich EIA. Collagen IV in the sample is bound simultaneously by a solid phase monoclonal antibody (IgG directed against human collagen IV ) and a monoclonal antibody-enzyme conjugate directed at different antigenic sites (Anti-collagen IV mouse Fab' conjugated to horseradish peroxidase).

These results in the collagen IV molecule being sandwiched between the solid phase and enzyme labeled antibodies. After removing unbound enzyme labeled antibody and sample, the plate is then incubated with enzyme substrate (Stabilized liquid TMB solution), resulting in the development of a colour. The colour developed is directly proportional to the amount of collagen IV in the sample. The development of colour is stopped by stop solution (sulphuric acid) and read immediately at 450nm using 630nm as reference.

For this assay we collect the urine samples in special collecting Tubes coated with urinary collagen IV stabilizer supplied with the kit.

#### Calculation of urinary type IV collagen: creatinine ratio ( $\mu\text{g} / \text{g}$ )

Urinary type IV collagen: creatinine ratio ( $\mu\text{g} / \text{g}$ )=urinary collagen IV ( $\mu\text{g/L}$ ) / urinary creatinine (g/L)

### RESULTS AND DISCUSSION

Statistical analysis was performed using *t*-student test to compare means. Correlation strength between parameters was determined by calculation of pearson's correlation coefficient. We considered  $P < 0.05$  as a significant level.

Microsoft Office Excel 2007 was used to process and analyze data.

The clinical characteristics of the study population are summarized in table 1.

The study group was: 164 individuals consisted of 25 healthy volunteers and 139 type 2 diabetic patients who had a wide range of duration of diabetes (5 months –35 years).

The mean  $\pm$  standard deviation (SD) of age in patients group was:  $57.8 \pm 9.2$  years (mean  $\pm$  standard deviation), (range 29-77 years) while it was in control group:  $44.7 \pm 8.4$  years, (range 30-65 years).

The mean  $\pm$  SD of weight in patients group was:  $85.2 \pm 15.8$  kg, (range 44-150 kg) while in control group was:  $47.8 \pm 13.3$  kg, (range 50-100 kg).



The mean  $\pm$  SD of systolic pressure in the control group was: 11.7  $\pm$  0.8 (mmHg), (range 10-12.5 mmHg) while in patients group was: 13.7  $\pm$  2 (mmHg), (range 8-19 mmHg).

And the mean  $\pm$  SD of diastolic pressure in the control group was: 7.5  $\pm$  0.5 (mmHg), (range 6-8.5 mmHg) but in patients group was 8.1  $\pm$  1.2 (mmHg), (range 5.3-12 mmHg).

**Table 1:** The clinical characteristics of the study population

	Control group	Type II diabetic patients group
Number	25	139
Duration of diabetes mellitus	–	5 months – 35 years
Age (years)	44.7 $\pm$ 8.4	57.8 $\pm$ 9.2
	30 - 65	29 – 77
Weight (kg)	47.8 $\pm$ 13.3	85.2 $\pm$ 15.8
	50 - 100	44 - 150
Systolic pressure (mmHg)	11.7 $\pm$ 0.8	13.7 $\pm$ 2
	10 - 12.5	8-19
Diastolic pressure (mmHg)	7.5 $\pm$ 0.8	8.1 $\pm$ 1.2
	6 - 8.5	5.3-12

Currently, the determination of urinary A: C ratio is the diagnostic test of diabetic nephropathy<sup>6</sup>, while the aim of our study was to evaluate the diagnostic value of urinary collagen IV in diabetic nephropathy as it is one of the normal constituents of the mesangium and glomerular basement membrane (GBM).<sup>4</sup>

So the patients group was divided according to the albumin: creatinine (A: C) ratio into three groups:

1. Group A: normoalbuminuria diabetic patients (n=79), A: C ratio < 30 mg/g.
2. Group B: microalbuminuria diabetic patients (n=39), A:C ratio 30-299 mg/g.
3. Group C: macroalbuminuria diabetic patients (n=21), A:C ratio  $\geq$  300 mg/g.<sup>7</sup>

The clinical markers measured in the study population are summarized in table.2, the mean  $\pm$  SD of A:C ratio in group A was : 8.1  $\pm$  9.0 mg/g ( range 0 – 28.98 mg/g), in group B was : 83.9  $\pm$  50.3 mg/g ( range 30 - 228.7 mg/g), in group C was : 911.5  $\pm$  774.4 mg/g ( range 300 - 3406.3 mg/g) and in the control group was : 5.5  $\pm$  4.4 mg/g (range 0 - 13.2 mg/g).

The mean  $\pm$  SD of fasting glucose in group A was : 195.8  $\pm$  71.8 mg/dl( range 82- 433 mg/dl) , in group B was : 229.7  $\pm$  82.5 mg/dl ( range 67 – 424 mg/dl), in group C was : 211  $\pm$  102.9 mg/dl ( range 62 – 466 mg/dl) and in the control group was : 100.7 $\pm$ 7.3 mg/dl ( range 88 – 110 mg/dl).

The mean  $\pm$  SD of HbA1 in group A was : 8.2  $\pm$  1.8 % ( range 4.5 – 13.4%) , in group B was : 8.8  $\pm$  2.5 % ( range

5.1- 15.5%), in group C was : 9.17  $\pm$  2.8% ( range 4.5- 15.4%) and in the control group was : 6.2  $\pm$  0.54 % ( range 4.5 -7%).

The mean  $\pm$  SD of serum creatinine in group A was : 0.86  $\pm$  0.20 mg/dl( range 0.54 –1.45 mg/dl) , in group B was : 0.86  $\pm$  0.24 mg/dl ( range 0.53 – 1.52 mg/dl), in group C was : 0.98  $\pm$  0.22 mg/dl ( range 0.71 – 1.46 mg/dl) and in the control group was : 0.85  $\pm$  0.17mg/dl (range 0.62 – 1.22 mg/dl).

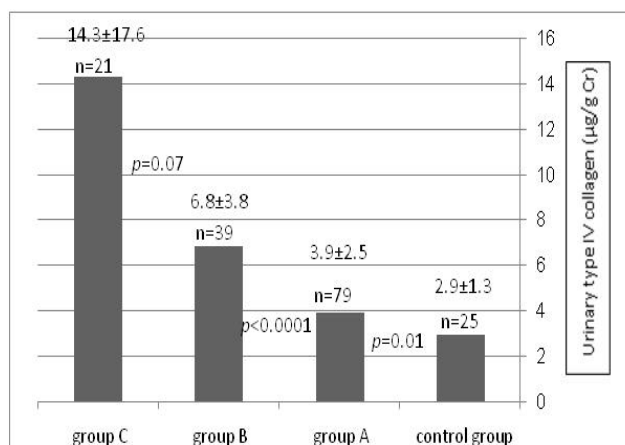
The mean  $\pm$  SD of eCcr in group A was : 111.2  $\pm$  35.1 ml/min (range 44.7- 200.4 ml/min), in group B was : 108.3  $\pm$  34.5 ml/min (range 39.6 – 196.3 ml/min), in group C was : 94.4  $\pm$  40.2 ml/min (range 44.6- 210.8 ml/min) and in the control group was :

108.8  $\pm$  25.4 ml/min (range 65.9 – 157.4 ml/min).

The mean  $\pm$  SD of urinary type IV collagen in group A was : 3.9  $\pm$  2.5  $\mu$ g/g Cr (range 0.4 – 13.5  $\mu$ g/g Cr), in group B was : 6.8  $\pm$  3.8  $\mu$ g/g Cr (range 1.5 – 15.8  $\mu$ g/g Cr), in group C was : 14.3  $\pm$  17.6  $\mu$ g/g Cr (range 1.7- 68.5  $\mu$ g/g Cr) and in the control group was : 2.9  $\pm$  1.3  $\mu$ g/g Cr (range 0.53 – 5.6  $\mu$ g/g Cr).

Urinary collagen IV (  $\mu$ g/g Cr) was significantly higher in group A , B and C than in control group (  $p=0.01$ , < 0.0001 and 0.007 respectively ). Also it was significantly higher in group B and group C than in group A (  $p < 0.0001$  and 0.01 respectively). There was no significant difference in urinary collagen IV excretion between group C and group B (  $p=0.07$  ) .

Urinary type IV collagen levels ( $\mu$ g/g of creatinine) in study population and differences between study groups are shown in (figure 1).

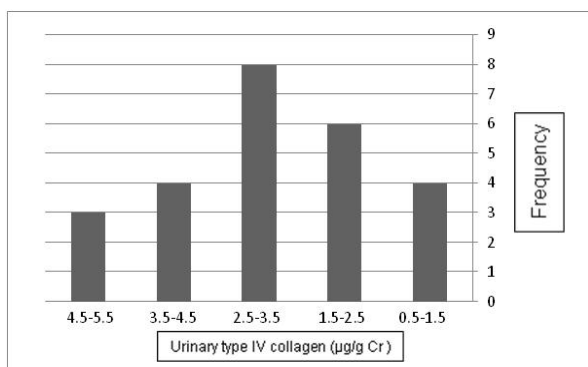


**Figure 1:** Urinary type IV collagen levels ( $\mu$ g/g of creatinine) in controls and diabetic patients and the differences between study groups.

Urinary type IV collagen levels ( $\mu$ g/g of creatinine) were distributed normally in the control group as shown in (figure 2).

**Table 2:** The clinical markers measured in the study population

	Control group	Group (A)	Group (B)	Group (C)
Number	25	79	39	21
Urinary A:C ratio (mg/g Cr)	5.5 ± 4.4 0 - 13.2	8.1 ± 9.0 0 – 28.98	83.9 ± 50.3 30 - 228.7	911.5±774.4 300 - 3406.3
Serum fasting glucose (mg/dl)	100.7±7.3 88 – 110	195.8 ± 71.8 82- 433	229.7 ± 82.5 67 – 424	211 ± 102.9 62 – 466
HbA1%	6.2 ± 0.54 4.5 -7	8.2 ± 1.8 4.5 – 13.4	8.8 ± 2.5 5.1- 15.5	9.17 ± 2.8 4.5-15.4
Serum creatinine (mg/dl)	0.85 ± 0.17 0.62 – 1.22	0.86 ± 0.20 0.54 –1.45	0.86 ± 0.24 0.53 – 1.52	0.98 ± 0.22 0.71 – 1.46
Estimated creatinine clearance (eCcr) according to Cockcroft-Gault (ml/min)	108.8 ± 25.4 65.9 – 157.4	111.2 ± 35.1 44.7- 200.4	108.3 ± 34.5 39.6 – 196.3	94.4 ± 40.2 44.6- 210.8
Urinary type IV collagen (µg/g Cr)	2.9 ± 1.3 0.53 – 5.6	3.9 ± 2.5 0.4 – 13.5	3.8 6.8 ± 1.5 – 15.8	14.3 ± 17.6 1.7- 68.5



**Figure 2:** The normal distribution of urinary type IV collagen levels (µg/g of creatinine) in the control group.

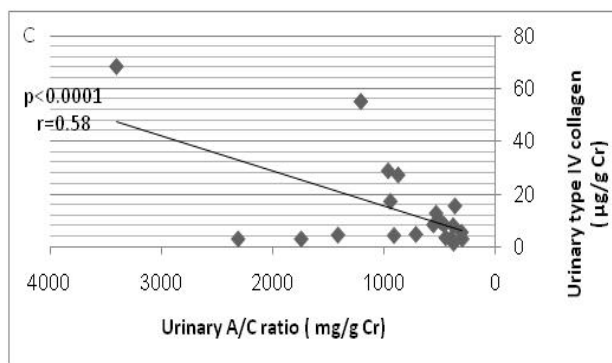
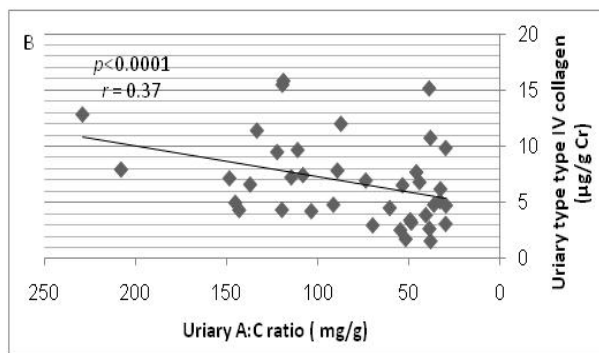
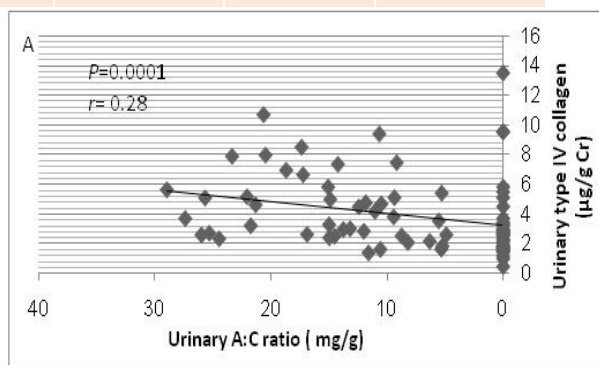
Urinary collagen IV showed significant positive correlation with A: C ratio values in group A ( $p=0.0001$ ,  $r=0.28$ ), group B ( $p<0.0001$ ,  $r=0.37$ ) and group C ( $p<0.0001$ ,  $r=0.58$ ) (figure 3).

Urinary collagen IV had significant inverse correlation with eCcr values in group A ( $p<0.0001$ ,  $r = -0.27$ ), while urinary A:C ratio values had less significant inverse correlation with eCcr values in the same group ( $p<0.0001$ ,  $r = -0.22$ ).

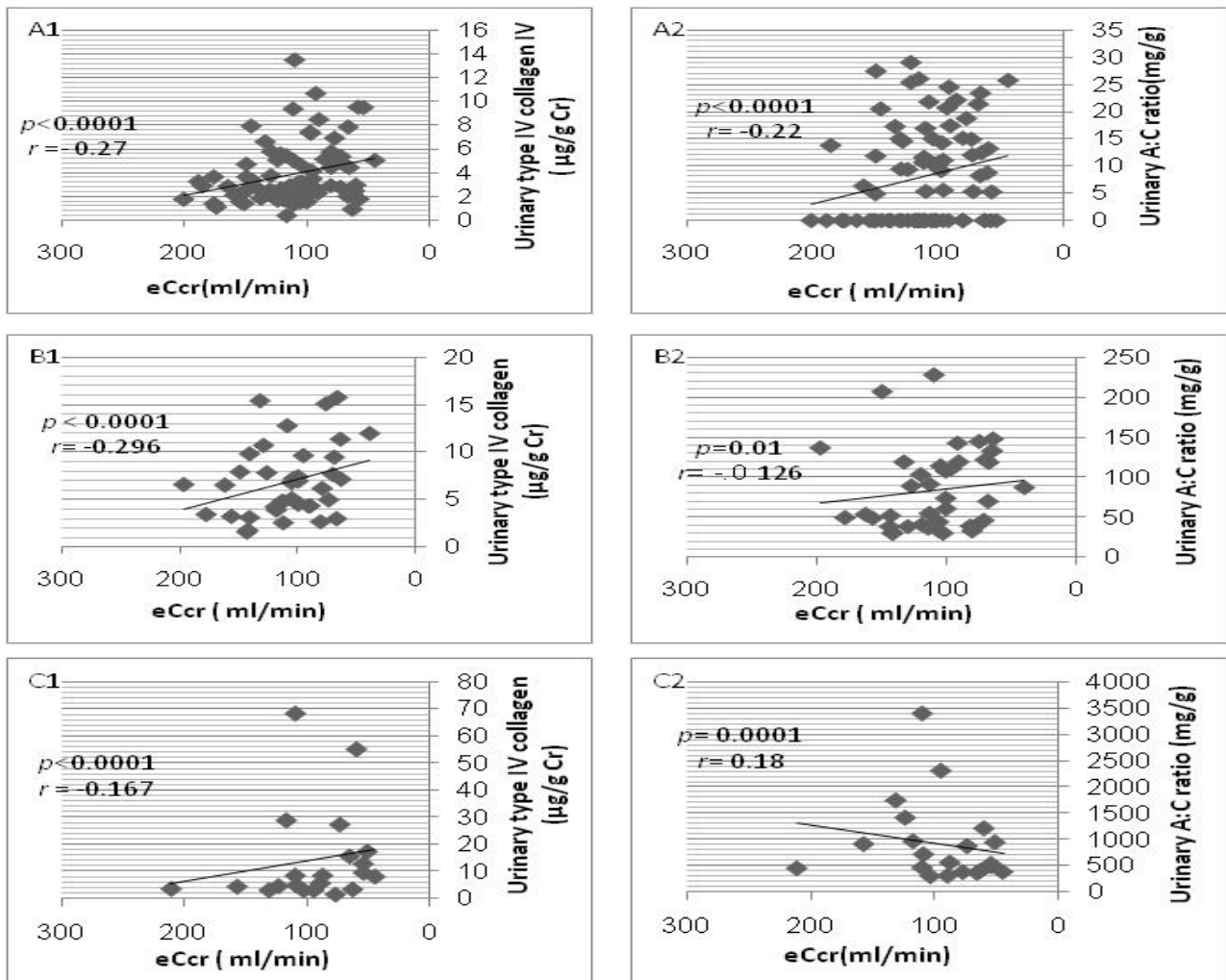
Urinary collagen IV had significant inverse correlation with eCcr values in group B ( $p<0.0001$ ,  $r = -0.296$ ), while urinary A:C ratio values had less significant inverse correlation with eCcr values in the same group ( $p=0.01$ ,  $r = -0.126$ ).

Urinary collagen IV had less significant inverse correlation with eCcr values in group C ( $p<0.0001$ ,  $r = -0.167$ ), while urinary A:C ratio values had significant positive correlation with eCcr values in the same group ( $p=0.0001$ ,  $r = 0.18$ ) (figure 4).

Urinary collagen IV showed no correlation with HbA1 levels in group A ( $p<0.0001$ ,  $r=0.06$ ), but had significant correlation with HbA1 levels in group B ( $p<0.0001$ ,  $r=0.29$ ) and group C ( $p=0.001$ ,  $r = 0.22$ ) (figure 5).

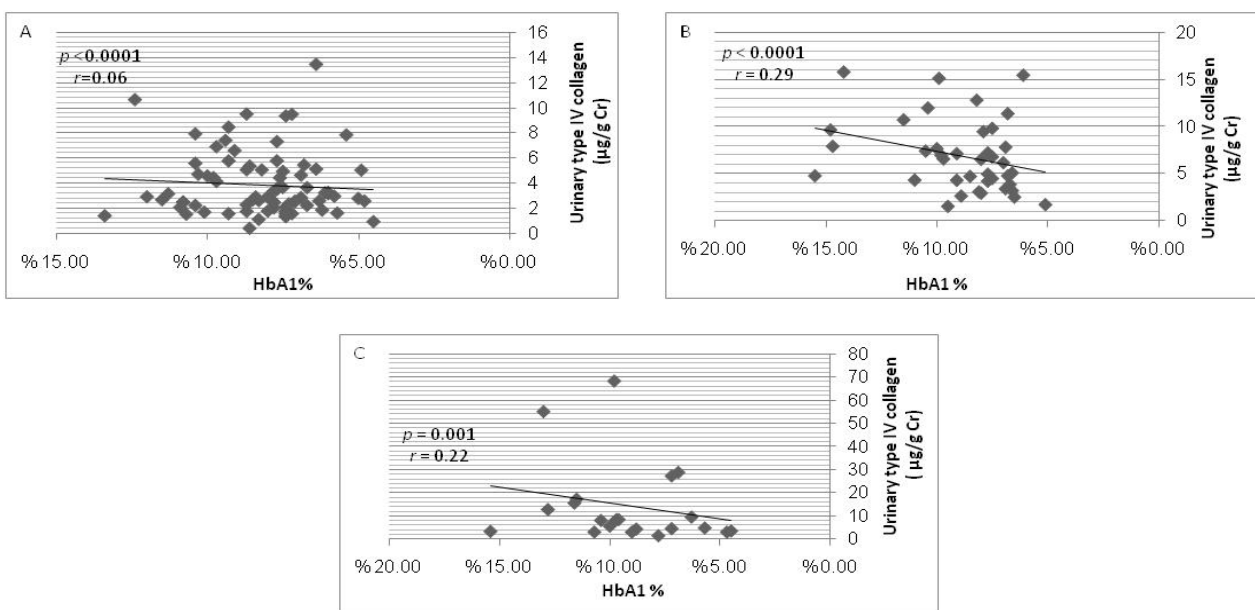


**Figure 3:** Correlation between urinary A:C ratio ( mg/g) and urinary type IV collagen (µg/g Cr) in group A (normoalbuminuric) (A), in group B (microalbuminuric) (B) and in group C (macroalbuminuric) (C) diabetes mellitus patients.



**Figure 4:** Correlation between eGCr ( ml/min) and urinary type IV collagen ( µg/g Cr) (A1), (B1) and (C1) in group A (normoalbuminuric), in group B (microalbuminuric) and in group C (macroalbuminuric) diabetic patients respectively .

And correlation between eGCr ( ml/min) and urinary A:C ratio (mg/g) ( A2),(B2) and (C2) in group A (normoalbuminuric), in group B (microalbuminuric) and in group C (macroalbuminuric) diabetic patients respectively .



**Figure 5:** Correlation between HbA1(%) levels and urinary type IV collagen (µg/g Cr) in group A (normoalbuminuric)(A), in group B (microalbuminuric)(B) and in group C (macroalbuminuric)(C) diabetic patients.

The characteristic kidney structural abnormalities that occur in diabetic nephropathy patients:

The increase in the thickness of glomerular basement membranes and accumulation of extracellular matrix components in the glomerulus.<sup>3</sup>

Type IV collagen is a normal constituent of the mesangium and glomerular basement membrane (GBM) and it is increased in diabetic kidney disease.<sup>4</sup>

Accumulation of the extracellular matrix is likely the main pathology that causes decreased renal function because it alters filtration function and interactions among mesangial cells, endothelial cells and podocytes, leading to mesangial expansion and glomerulosclerosis.<sup>8</sup>

The main findings of this study are: that urinary type IV collagen excretion is increased in normoalbuminuric patients more than control group and it is increased with increased urinary albumin excretion, the same results were found in Shin-ichi Araki *et al* study in Japan<sup>9</sup> and Pavai Sthaneshwar and Siew-Pheng Chan study in Malaysia<sup>10</sup> urinary type IV collagen levels are increased (obviously than increased urinary albumin excretion) with decreased eCcr levels calculated by Cockcroft-Gault formula, definitely with the decline of the filtration function in diabetic patients, the same results were found in Margo P. Cohen *et al* study in the USA<sup>11</sup> and urinary type IV collagen amounts are increased with increased HbA1 levels in microalbuminuric and macroalbuminuric diabetic patients but we did not correspond in this result with any study, may be the increase in type IV collagen amounts is based on metabolic effects - like hyperglycemia- in these stages micro albuminuria and macro albuminuria more than hemodynamic effects .

### CONCLUSION

So urinary collagen IV excretion may be a better indicator than the A:C ratio of the decline in filtration function in diabetic nephropathy.

We can consider urinary type IV collagen as an early, sensitive and non invasive marker for diabetic nephropathy, and it may be also as an indicator of the progression of diabetic nephropathy.

Urinary type IV collagen levels may be influenced by blood glucose control in advanced stages of diabetic nephropathy.

We recommend:

1. Measuring urinary type IV collagen levels in type II diabetic patients and comparing its levels with other markers of diabetic nephropathy.
2. Studying its levels in the ESRD (end stage renal disease) stage and comparing them with its levels in the previous stages of diabetic nephropathy.

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