

# Evaluation of early biomarkers of renal dysfunction in diabetic patients

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## ABSTRACT

**الأهداف:** تقييم اثنين من الأنزيمات الأنبوبية الكلوية، (uNGAL) والبولون (N-acetyl-beta-d-glucosaminidase (uNAG) ومصل سيستاتين C كمؤشرات حيوية لتشخيص المبكر للمرحلة المبكرة من اعتلال الكلية السكري للمرضى الذين يعانون من مرض السكري من النوع الثاني .

**المنهجية:** تمت دراسة الحالات في مستشفى جامعة الملك عبد العزيز، جدة، المملكة العربية السعودية خلال الفترة من مايو 2017م-2018م، وأجريت على 86 مريضاً ممن يعانون من السكري النوع الثاني خلال عام 2017م. تم تصنيف المرضى وفقاً لنسبة مصل الألبومين/الكرياتينين (ACR) إلى 3 مجموعات؛ مجموعة ضابطة مع ACR أقل من 30 مجم/جم من الكرياتينين، ومجموعة من قليلة الارتفاع مع ACR: 30-299 ملغ/جم من الكرياتينين ومجموعة عالية جداً مع ACR أقل من أو يساوي 300 ملغ/جم. تم تجنيد البالغين الأصحاء كمجموعة مراقبة. تم قياس انزيمات البول uNGAL، uNAG، ومصل السيستاتين C.

**النتائج:** بالمقارنة مع التحكم الصحي، فإن المجموعة الضابطة من مرضى السكري يفرضون مستويات أعلى بكثير من uNGAL قيمة إحصائية ( $p < 0.001$ ). بالإضافة إلى ذلك، لوحظت مستويات مرتفعة من مستويات uNGAL و uNAG ومصل السيستاتين C في مجموعات متوسطة الارتفاع وعالية الارتفاع عند مقارنتها بالمجموعات الضابطة (normoalbuminuric) قيمة إحصائية ( $p > 0.001$ ). أظهر كل من uNGAL و uNAG ومصل السيستاتين C وجود علاقة إيجابية مع صيام نسبة الجلوكوز في الدم (FBG) و معدل السكر التراكمي ومدة مرض السكري واليوريا والكرياتينين و ACR.

**الخلاصة:** لقد أوضحت النتائج أن uNGAL يمكن أن يكون علامة حيوية حساسة لاختلال وظيفي كلوي مبكر في مرضى السكري في حين أن uNAG و مصل السيستاتين C يكون لهما قيمة تنبؤية.

**Objectives:** To evaluate 2 renal tubular enzymes; urinary neutrophil gelatinase-associated lipocalin (uNGAL), and urinary N-acetyl-beta-D-glucosaminidase (uNAG), and serum Cystatin C as candidate biomarkers for early diagnosis of early stage of diabetic nephropathy (DB) in patients with type 2 diabetes mellitus (T2DM).

**Methods:** This cross-sectional study was carried out at King Abdulaziz University Hospital (KAUH), Jeddah, Saudi Arabia during the period between May 2017 and May 2018 and was conducted on 86 patients with T2DM. Patients were

classified according to their albumin/creatinine ratio (ACR) into 3 groups; a normal albuminuria group with ACR  $< 30$  mg/g creatinine, a moderately increased albuminuria group with ACR: 30-299 mg/g creatinine, and a severely increased albuminuria group with ACR  $\geq 300$  mg/g. Healthy adults were recruited as a control group. Urine uNGAL, uNAG, and serum Cystatin C were measured in all patients.

**Results:** Compared with healthy control, diabetic patients with normal albuminuria excreted significantly higher levels of uNGAL ( $p < 0.001$ ). In addition, significantly elevated uNGAL, uNAG and cystatin C levels were observed in moderately increased albuminuria and severely increased albuminuria groups when compared to the control and normoalbuminuric groups ( $p < 0.001$ ). urinary neutrophil gelatinase-associated lipocalin, urinary N-acetyl-beta-D-glucosaminidase and Cystatin C showed a positive correlation with fasting blood glucose (FBG), HbA1c, duration of diabetes, urea, creatinine, and ACR.

**Conclusion:** Our results indicated that uNGAL could be a sensitive biomarker for early renal dysfunction in diabetic patients while uNAG and serum Cystatin C might have prognostic value.

**Keywords:** type 2 diabetes, diabetic nephropathy, NGAL, NAG, cystatin C

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A major microvascular complication of diabetes mellitus (DM) is diabetic nephropathy (DN), which leads to end-stage renal disease (ESRD) and is associated with increased cardiovascular mortality.<sup>1</sup> Moderately increased albuminuria (formerly called microalbuminuria) can be described as an increase in the level of albumin in urine below the clinical albuminuria levels and is considered an early sign of DN. At the stage of moderately increased albuminuria with euglycemic control, DN can be reversible. For this reason, it is important to detect nephropathy at or prior to this stage.<sup>2</sup> In order to detect early DN, many biomarkers are used and their appearance in urine and blood corresponds to different mechanisms or structural damage in DN. Therefore, those proteins are divided into biomarkers of glomerular injury, tubular injury, or endothelial damage. An example of these proteins are neutrophil gelatinase-associated lipocalin (NGAL), N-acetyl-beta-D-glucosaminidase (NAG), and Cystatin C.<sup>3</sup> N-acetyl-beta-D-glucosaminidase is an enzyme that belongs to the lipocalin family and is found in a number of tissues, such as the kidney. Hence, there are many reports of NGAL in acute kidney injury (AKI).<sup>4</sup> In chronic kidney disease (CKD), NGAL is reported to be a novel, independent biomarker of the disease severity, and progression.<sup>5,6</sup> N-acetyl-beta-D-glucosaminidase is an enzyme present in lysosomes and originates from cells of the proximal renal tubule. It is a high molecular weight protein (130000 to 140000 Daltons); therefore, it does not filter through the glomerulus. Consequently, the urinary excretion of NAG is relatively constant with minor diurnal variations and its level increases in urine exclusively due to injury in proximal tubular cells. In addition, it is stable against changes in temperature and pH.<sup>7,8</sup> Moreover, another biomarker is Cystatin C which is synthesized by all nucleated cells and secreted in the plasma. Due to its low molecular weight the protein is filtered by the glomerulus and then reabsorbed and catabolized by the proximal tubule, thus its serum levels can act as a marker for glomerular filtration rate (GFR).<sup>9</sup>

Renal dysfunction is a devastating disease that affects a rising number of patients with diabetes. It is very hard to get a cure once the disease has been diagnosed. For this reason, early diagnosis of DN has become an interesting area and indeed different proteins have been

proposed as biomarkers. However, some difficulties still remain, for example discovering efficient biomarkers to diagnose the disease prior to the appearance of clinical evidence.

Therefore, the aim of our study was to evaluate the 2 renal tubular enzymes: uNGAL and uNAG, and serum Cystatin C as biomarkers of renal dysfunction in T2DM. These tubular damage markers have been extensively investigated for predicting the occurrence of AKI;<sup>10</sup> however, little research has been carried out in patients with CKD. In this study, we investigated the serum Cystatin C and urinary levels of the NAG and NGAL in diabetic patients and nondiabetic control subjects to evaluate their potential as biomarkers of renal dysfunction in T2DM in the Saudi population.

**Methods.** This is a cross-sectional study involving 116 adults of both genders, age between 40 -70 years. Participants were selected from the medical clinic at King Abdulaziz University Hospital (KAUH), Jeddah, Kingdom of Saudi Arabia, between November 2017 and May 2018. The number of patients in each group was estimated prior to conducting the study based on various previous literature and after an estimation of the patient load at KAUH clinics. Recruitment of participants took place until similar numbers of patients were achieved in all studied groups. Subjects visiting the diabetes clinic were automatically selected if the patient fits the inclusion and exclusion criteria mentioned below.

All subjects were provided with written informed consent to provide needed information, undergo clinical examination, and provide blood and urine samples. The Research Ethical Committee Board of KAUH approved the consent form. Cases (N=86) were individuals diagnosed with T2DM according to the American Diabetes Association (ADA) criteria (diagnosed at an HbA1c of  $\geq 6.5\%$ ) based on our institution protocol, and suffered from diabetes for more than 5 years. Patients were further classified according to the albumin/creatinine ratio (ACR), into 3 groups: normal albuminuria group with ACR  $>30$  mg/g creatinine (n=26), moderately increased albuminuria group with ACR 30-299 mg/g creatinine (n=30) and severely increased albuminuria group with ACR  $>300$  mg/g creatinine (n=30). Healthy subjects with matched age and gender were included as a control group (n=30). Each subject was asked about their age, medical history, previous medication and duration of DM. Moreover, the BMI and blood pressure measurements were taken for all subjects. Patients with urinary tract infection, malignancies, liver disease, thyroid gland dysfunction,

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uncontrolled hypertension, uncontrolled dyslipidemia, and cardiovascular disease were excluded. We aimed to eliminate any biases since these diseases were found in various studies to alter the expression of the studied markers.<sup>11-15</sup>

Venous blood samples were collected from all subjects after an overnight fast (at least 12 hours). Routine biochemical tests were carried out immediately. At least 2 ml of serum was kept and stored at -80°C for further analysis.

A morning urine sample was collected in sterile urine container, centrifuged for 20 minutes and the supernatant was collected then aliquoted and stored at -80°C for NAG and NGAL analysis.

Routine biochemical tests were carried out immediately on the same day of sample collection in the biochemistry laboratory of KAUH by using enzymatic test and bichromatic rate technique for fasting blood glucose (FBG), total cholesterol (TC), triglyceride (TG), high-density lipoprotein cholesterol (HDL-C), low density lipoprotein cholesterol (LDL-C), serum creatinine (SCr) and blood urea nitrogen (BUN), immunochemical reaction for (microalbumin), and turbidimetric inhibition immunoassay for hemoglobin A1c (A1C). All the tests were carried out using autoanalyzer (Dimension Vista System, Siemens Healthcare diagnostic Inc., Newark, USA). The concentration of FBG, TC, TG, HDL-C, LDL-C and BUN were reported in mmol/l while SCr was reported in  $\mu\text{mol/l}$  and A1C was reported in percentage. Spot urine albumin/ACR obtained under standardized conditions according to the National Kidney Foundation recommendations.<sup>16</sup> Albumin/creatinine ratio was calculated based on the below equation:

$$\text{ACR (mg/g creatinine)} = \text{albumin/creatinine}$$

Positive ACR results were based on the mean of 3 different samples in one year.<sup>17</sup> Glomerular filtration rate was calculated based on the modification of diet in renal disease (MDRD) study equation:

$$\text{GFR (mL/min/1.73 m}^2\text{)} = 186 \times \text{SCr}^{-1.154} \times \text{age}^{-0.203} \times (0.742 \text{ if female}) \times (1.212 \text{ if black})$$

**Determination of uNAG, uNGAL and serum Cystatin C.** Urinary N-acetyl-beta-D-glucosaminidase, uNGAL and serum Cystatin C concentrations were determined by using human enzyme linked immunosorbent assay (ELISA) kits purchased from Elabscience (Elabscience Biotechnology Co.,Ltd, USA).

**Statistical analysis.** All the reported data was recorded and analyzed by Statistical Package for Social Science version 21 (Armonk, NY: IBM Corp.) Data was described as mean  $\pm$  standard deviation (SD). Significant comparison between groups was conducted using an ANOVA test. Post Hoc Least Significant Difference (LSD) test was performed to determine the intragroup significance. Pearson's correlation was used to assess correlation between markers and other tested variables. A  $p < 0.05$  was considered statistically significant.<sup>7</sup>

**Results. The biochemical and anthropometric differences between T2DM groups and healthy control subjects.** Anthropometric and laboratory parameters comparison between the different groups are represented in **Table 1**. The mean age  $\pm$  SD of T2DM groups; normal albuminuria was  $53 \pm 5$ , moderately increased albuminuria was  $53 \pm 8$ , and severely increased albuminuria was  $56 \pm 8$  years, while for healthy control it was  $51 \pm 8$  years. As expected, the moderately increased albuminuria ( $15 \pm 4$ ) and severely increased albuminuria ( $13 \pm 5$ ) diabetic groups suffered diabetes for longer durations in comparison to the normal albuminuria diabetic group ( $10 \pm 4$ ). The mean levels of FBG and A1C were significantly higher in all the 3 diabetic groups as compared to the control ( $p < 0.001$ ). A significant higher systolic blood pressure levels were observed in moderately increased albuminuria and severely increased albuminuria diabetic groups as compared to the control ( $p < 0.01$ ). However, no statistically significant difference was observed in terms of the diastolic blood pressure levels between groups (**Table 1**).

Similarly, there were no significant differences in BMI between all groups. Additionally, the levels of serum urea and creatinine were significantly higher in diabetic groups than that observed in control subjects ( $p < 0.001$ ). On the contrary, the mean levels of GFR were significantly lower in all of 3 diabetic groups as compared to the control group ( $p < 0.001$ ). The levels of cholesterol ( $p < 0.01$ ) and triglycerides ( $p < 0.05$ ) in the diabetic groups were significantly higher than that observed in control. On the other hand, HDL-C and LDL-C levels did not show any significant differences between groups.

**Urinary neutrophil gelatinase-associated lipocalin, uNAG and Cystatin C in T2DM groups and healthy control subjects.** Urinary neutrophil gelatinase-associated lipocalin levels were significantly increased in the severely increased albuminuria group in comparison to all 3 groups; moderately increased,

**Table 1** - Comparison between the different studied groups regarding anthropometric and laboratory parameters.

Variables	Control group n=30	Normal albuminuria group n=26	Moderately increased albuminuria group n=30	Severely increased albuminuria group n=30	P-value
Age (years)	51 ± 8	53 ± 5	53 ± 8	56 ± 8	-
<b>Gender (%)</b>	15 (50)	10 (39)	17 (57)	16 (53)	-
Male	15 (50)	16 (61)	13 (43)	14 (47)	
Female					
SBP (mm/Hg)	129.5 ± 19	136 ± 18	143 ± 21	147.3 ± 17	0.003*
DBP (mm/Hg)	69.1 ± 10	69 ± 10	71.1 ± 10	74.3 ± 10	0.205 <sup>‡</sup>
BMI (Kg/m <sup>2</sup> )	29.6 ± 3.7	31.1 ± 4.4	32.1 ± 5.7	30.5 ± 4.6	0.102 <sup>‡</sup>
Duration of diabetes (years)	NA	10 ± 4	13 ± 5	15 ± 4	<0.001*
FBG (mmol/l)	5.1 ± 0.2	8.5 ± 4.1	9.6 ± 5.7	9.7 ± 3	<0.001*
A1C (%)	5.3 ± 0.4	7.9 ± 1.3	9.1 ± 2.3	9.6 ± 1.4	<0.001*
Urea (mmol/l)	4 ± 1	4.4 ± 1.3	5.6 ± 2.5	8.1 ± 6	<0.001*
SCr (µmol/l)	65.6 ± 20.2	72.7 ± 18.4	84.7 ± 27.2	166.7 ± 58.6	<0.001*
GFR (mL/min/1.73 m <sup>2</sup> )	103.5 ± 25.7	89.1 ± 29.5	72.6 ± 24.6	38.5 ± 21.4	<0.001*
TC (mmol/l)	4.1 ± 0.6	4.1 ± 0.8	4.3 ± 0.8	4.9 ± 1.5	0.008*
TGs (mmol/l)	1.3 ± 0.5	1.5 ± 1	1.9 ± 1	2.1 ± 1.5	0.030*
HDL-C (mmol/l)	1.5 ± 0.2	1.3 ± 0.4	1.3 ± 0.3	1.4 ± 0.7	0.063 <sup>‡</sup>
LDL-C (mmol/l)	2.8 ± 0.4	2.8 ± 0.8	3 ± 0.8	3.2 ± 1.1	0.099 <sup>‡</sup>

Data presented as mean ± SD. †One way ANOVA between all groups. \*significant values, NA: not applicable, BMI: body mass index, SBP: systolic blood pressure, DBP: diastolic blood pressure, FBG: fasting blood glucose, A1C: glycated hemoglobin, SCr: serum creatinine, GFR: Glomerular filtration rate, TC: total cholesterol, TGs: triglycerides, HDL-C: high density lipoprotein cholesterol, LDL-C: low density lipoprotein cholesterol

**Table 2** - Comparison between the different studied groups regarding ACR, uNGAL, uNAG, and Cystatin C.

Variables	Control group n=30	Normal albuminuria group n=26	Moderately increased albuminuria group n=30	Severely increased albuminuria group n=30
ACR (mg/gCr)	7 ± 6.7	17 ± 7	145.6 ± 82.5 <sup>*,†</sup>	562.2 ± 237.9 <sup>*,†,‡</sup>
uNGAL (ng/ml)	3.5 ± 0.9	4.2 ± 1.1*	5.1 ± 1.5 <sup>*,†</sup>	7.5 ± 0.8 <sup>*,†,‡</sup>
uNAG (ng/ml)	26.4 ± 7.7	26.7 ± 7.3	41.1 ± 11.7 <sup>*,†</sup>	48.8 ± 11.3 <sup>*,†,‡</sup>
Cystatin C (ng/ml)	641.1 ± 28.9	676.6 ± 60.4	969.1 ± 124.7 <sup>*,†</sup>	1653.3 ± 446.2 <sup>*,†,‡</sup>

Data presented as mean ± SD. P-value calculated by One-way ANOVA post hoc LSD tests (\*p<0.01 versus control; †p<0.01 moderate and severe albuminuria versus normal albuminuria, ‡p<0.01 severe albuminuria versus moderate albuminuria). ACR: albumin /creatinine ratio, uNGAL: urinary neutrophil gelatinase-associated lipocalin, uNAG: urinary N-acetyl-beta-D-glucosaminidase

**Table 3** - Correlation of Cystatin C, uNGAL uNAG and other biochemical variables in diabetic patient with nephropathy.

Variables	Cystatin C		uNGAL		uNAG	
	r	P-value	r	P-value	r	P-value
Cystatin C (ng/ml)			0.76**	<0.001	0.57**	<0.001
uNAG (ng/ml)	0.57**	<0.001	0.51**	<0.001		
uNGAL (ng/ml)	0.76**	<0.001			0.51**	<0.001
ACR (mg/g)	0.80**	<0.001	0.73**	<0.001	0.56**	<0.001
Duration (years)	0.52**	<0.001	0.63**	<0.001	0.43**	<0.001
FBG (mmol/l)	0.28**	0.002	0.31**	0.001	0.26*	0.005
A1C (%)	0.49**	<0.001	0.48**	<0.001	0.44**	<0.001
Urea (mmol/l)	0.48**	<0.001	0.49**	<0.001	0.30**	0.001
SCr (μmol/l)	0.78**	<0.001	0.63**	<0.001	0.47**	<0.001
GFR (mL/min/1.73 m <sup>2</sup> )	-0.66**	<0.001	-0.56**	<0.001	-0.49**	<0.001

r: Pearson correlation coefficient, \*\*correlation is significant at the 0.01 level (2-tailed), \*correlation is significant at the 0.05 level (2-tailed).  
 ACR: albumin /creatinine ratio, uNGAL: urinary neutrophil gelatinase-associated lipocaline, uNAG: urinary N-acetyl-beta-D-glucosaminidase,  
 FBG: fasting blood glucose, A1C: glycated hemoglobin, SCr: serum creatinine, GFR: glomerular filtration rate.

normal albuminuria, and control group ( $p < 0.001$ ). In addition, uNGAL levels were significantly increased in the moderately increased albuminuria group ( $p < 0.01$ ) in comparison to normal albuminuria and control group ( $p < 0.001$ ). Interestingly, the levels of uNGAL were significantly increased in the normal albuminuria group in comparison to control group ( $p < 0.05$ ), indicating that uNGAL can be an early sensitive marker for DN in T2DM (Table 2). Similar to uNGAL, uNAG levels were significantly increased in the severely increased albuminuria group in comparison to all 3 groups; moderately increased albuminuria ( $p < 0.01$ ), normal albuminuria ( $p < 0.001$ ), and control group ( $p < 0.001$ ). In addition, uNAG levels were significantly increased in the moderately increased albuminuria group in comparison to normal albuminuria and control group ( $p < 0.001$ ).

However, in contrary with uNGAL, uNAG levels were not significantly increased in the normal albuminuria group in comparison to control group (Table 2). Serum Cystatin C levels were also significantly increased in the severely increased albuminuria group in comparison to all 3 groups; moderately increased albuminuria, normal albuminuria, and control group ( $p < 0.001$ ). In addition, serum Cystatin C levels were significantly increased in the moderately increased albuminuria group in comparison to normal albuminuria and control group ( $p < 0.001$ ). However, similar to uNAG, serum Cystatin

C levels were not significantly increased in the normal albuminuria group in comparison to control group (Table 2).

**Correlation between uNGAL, uNAG and Cystatin C and other variables.** To evaluate the correlation between our studied markers and other variables we used Pearson correlation coefficient (Table 3). Our correlation studies revealed significant positive correlations between uNGAL and FBG ( $r = 0.310$ ,  $p < 0.001$ ); A1C ( $r = 0.475$ ,  $p < 0.001$ ); disease duration ( $r = 0.631$ ,  $p < 0.001$ ); serum creatinine ( $r = 0.634$ ,  $p < 0.001$ ); urea ( $r = 0.486$ ,  $p < 0.001$ ) and ACR ( $r = 0.732$ ,  $p < 0.001$ ).

Similarly, there was a significant positive correlation between uNAG and FBG ( $r = 0.258$ ,  $p < 0.01$ ); A1C ( $r = 0.441$ ,  $p < 0.001$ ); disease duration ( $r = 0.432$ ,  $p < 0.001$ ); serum creatinine ( $r = 0.473$ ,  $p < 0.001$ ); urea ( $r = 0.297$ ,  $p < 0.001$ ) and ACR ( $r = 0.557$ ,  $p < 0.001$ ). There was also a significant positive correlation between Cystatin C and FBG ( $r = 0.281$ ,  $p < 0.01$ ); A1C ( $r = 0.491$ ,  $p < 0.001$ ); disease duration ( $r = 0.515$ ,  $p < 0.001$ ); serum creatinine ( $r = 0.784$ ,  $p < 0.001$ ); urea ( $r = 0.480$ ,  $p < 0.001$ ) and ACR ( $r = 0.804$ ,  $p < 0.001$ ). While as expected, GFR showed a significant negative correlation with uNGAL ( $r = -0.585$ ,  $p < 0.001$ ); uNAG ( $r = -0.488$ ,  $p < 0.001$ ) and Cystatin C ( $r = -0.662$ ,  $p < 0.001$ ). Finally, we were interested in evaluating any correlation between the studied markers. Correlation studies revealed that uNGAL, uNAG, and Cystatin C

showed significant positive correlations between each other. There was a significant positive correlation between uNGAL and uNAG ( $r=0.514$ ,  $p<0.001$ ); and also between uNGAL and Cystatin C ( $r=0.761$ ,  $p<0.001$ ) (Table 3). In addition, there was a significant positive correlation between uNAG and Cystatin C ( $r=0.571$ ,  $p<0.001$ ).

**Discussion.** In our study, we evaluated uNGAL, uNAG and Cystatin C as early biomarkers for renal dysfunction in T2DM in Saudi patients. Albuminuria and serum creatinine have been considered for a long period as the gold standard biomarkers for DN onset and progression.<sup>18</sup> However, the limitations associated with these biomarkers have led to the need for earlier and more sensitive and specific biomarkers with greater detectability.

In our study, uNGAL levels were significantly elevated in all diabetic groups, especially in moderately increased albuminuria and severely increased albuminuria groups. These findings were in agreement with a previous cohort study carried out on 70 T2DM patients with different stages of DN classified according to the ACR.<sup>19</sup> Many recent studies were consistent with our results.<sup>20-23</sup> For example, in a recent cohort study of 146 Chinese T2DM patients, with a disease duration  $\geq 6$  years, uNGAL was higher in T2DM patients with a normal ACR,<sup>23</sup> which like our finding supports the use of uNGAL as a marker of early detection of nephropathy before the development of moderately increased albuminuria.<sup>22</sup> The rise of uNGAL in normal albuminuria patients might indicate early glomerular and tubular injuries; therefore, uNGAL can act as a novel biomarker for renal impairment. Conversely, another explanation could be that uNGAL is a specific biomarker for hyperglycemia without kidney injury; however, this would require further experiments to determine.<sup>23</sup> Overall, these results, together with our results, suggest that tubular injury may precede glomerular injury and that uNGAL could be used as an early biomarker for renal diseases in diabetics. This also suggests that tubular dysfunction in early DN might not be secondary to albuminuria, but a consequence of metabolic and hemodynamic stress that is associated with chronic hyperglycemia.<sup>24</sup> However, it is important to mention that some studies did not show the same level of sensitivity for uNGAL, as it was not significantly elevated in the normal albuminuria diabetic patients.<sup>25,26</sup> For example, data of a cross-sectional study which was conducted on T2DM patients enrolled from general hospitals in Riyadh, demonstrated that uNGAL levels were significantly increased in patients with moderately increased albuminuria and severely increased albuminuria but not in normal albuminuria

patients.<sup>25</sup> This variation from our results might reflect the differences in sample size but could also be due to different therapeutic regimens for glycemic control that would be expected between different centres. It should be noted that some diseases can effect the expression of the studied tubular marker and thus our study aimed to exclude patients with the described diseases. For example, urinary tract infections can lead to false positive results of uNGAL since the NGAL is a protein present in neutrophil that are elevated in urinary tract infections.<sup>12</sup> Further evidences indicate that NGAL can have an oncogenic role through inducing inflammation and by regulating cell growth and cell adhesion in cells.<sup>27</sup>

Finally, correlation analysis in our current study showed that uNGAL correlated with the progression of the disease since its excretion levels in urine had a significant positive correlation with ACR. This concludes that uNGAL can be used as a marker to stratify DN into different stages. These results were comparable to what has been observed in a previous study in which uNGAL showed a positive correlation with albuminuria suggesting that the former correlates with the severity of renal involvement.<sup>24</sup>

In this study, we found that uNAG was significantly increased in moderately increased albuminuria and severely increased albuminuria diabetic groups compared to the control group. In our study, uNAG was not increased significantly in the normal albuminuria group compared to the control group. Similar to our results, other researchers have suggested that uNAG did not have any clinical significance as an early biomarker of DN.<sup>2</sup> Some patients in our study may be exposed to drugs, such as angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs) which are known to reduce albuminuria and tubular biomarkers and thus, could be the reason of this finding.<sup>28</sup> However, the literature remains controversial and some studies have suggested a role for uNAG as an early biomarker of DN. For example, Nauta et al, 2011 found that uNAG levels were increased 9 folds in diabetics with normal albuminuria in comparison to control. In addition, it was gradually elevated at different stages of albuminuria.<sup>20</sup> The authors explained their results by showing that uNAG was associated with diabetes-related factors in diabetics with normal albuminuria, such as the duration of diabetes and BMI. It is believed that an increase in uNAG indicates tubular damage since the enzyme can only be secreted by cells of the proximal tubule and is not filtered by the glomerulus. Together with a decreased GFR an increase in uNAG would indicate therefore a glomerulotubular damage.<sup>29</sup> Indeed, in the present study we found that the uNAG levels showed a negative correlation with

GFR, which is in agreement with another large cross-sectional study that was conducted on 592 patients with diabetes.<sup>30</sup> There are several plausible explanations for the association between high glucose levels in plasma or urine and increased uNAG excretion. One explanation is that uNAG is an enzyme involved in carbohydrate metabolism. Thus, with continuous exposure of proximal tubules to high glucose, it is expected that NAG secretion would increase to metabolise the high levels of glucose.<sup>31</sup> However, another explanation could be related to the nephrotoxic effect of glycated end products on proximal tubules. This can contribute to tubular injury associated with an increase in NAG secretion.<sup>30</sup>

Our results showed that serum Cystatin C levels were significantly increased in diabetics patients with moderately increased albuminuria and severely increased albuminuria in comparison with the control group. This is in agreement with earlier studies.<sup>19</sup> However, in our study, there was no statistical significance between the Cystatin C levels in patients with normal albuminuria and control group. These results are consistent with the results of a recent Saudi study which included 200 T2DM patients classified according to ACR, in which serum Cystatin C was not significantly different in diabetics with normal albuminuria when compared to the control group.<sup>32</sup> Contrary to our results, a cross-sectional study by Takir et al,<sup>33</sup> reported that Cystatin C might play a significant role in the development of early DN and that its level was raised significantly in normal albuminuria diabetic patients with GFR <60 ml/m/1.73 m<sup>2</sup>. However, this discrepancy with our results could be explained by the different group classification since their patients were classified according to the GFR instead of ACR. Indeed, in our study, Cystatin C showed a significant positive correlation with ACR and a significant negative correlation with GFR and these results are similar to what was found by Javanmardi et al,<sup>34</sup> in which they found that serum Cystatin C levels increased significantly in association with decreasing GFR in 126 T2DM patients.

The current study has several strengths. We evaluated 3 various biomarkers in different stages of DN for better assessment of renal function. In our study, we matched control group for age and gender thus eliminating confounders that can interfere with the results. In addition, we had an exclusion criteria that excludes diseases that can interfere with the production of the studied markers.

Despite the significant conclusions that were obtained from our data, some of the limitations associated with the study must be stated. First of all, the sample size

might not be sufficient to determine the reliability and generalizability of the 3 assessed biomarkers in expressing the degree of renal impairment in DN. We believe that more studies, and on a larger scale, will be needed to confirm our results. Our results are also limited to our Saudi patient population, thus limiting generalizability since other factors might be playing a role in the increased biomarkers. Finally, some of our patients were under therapeutic control from other conditions such as hypertension and dyslipidemia, and this may lead to some bias due to the protective role of these drugs.

In conclusion, in the present study, uNGAL was elevated in T2DM patients with normal albuminuria, suggesting that the sensitivity of uNGAL was better than uNAG and serum Cystatin C in early detection of nephropathy in these patients. As a result, uNGAL could be considered as a potential, valuable, and non-invasive marker that could, with further studies, prove to be an independent predictor for the estimation of renal dysfunction in diabetes in our Saudi population. However, despite several studies on uNGAL it has not yet reached its clinical potential, and it is not yet used routinely in clinical care. We therefore recommend to conduct further studies on a larger scale to generalize the results and to eliminate the effect of any confounding factors. In addition, an enhanced understanding of the exact mechanism behind the rise of uNGAL is required; for example whether uNGAL is a specific biomarker for hyperglycemia without kidney injury would be an interesting question to address. Our results also suggest that uNAG and serum Cystatin C can play a role during the diagnosis and the follow up of T2DM patients with the onset of moderately increased albuminuria. Thus, together with uNGAL, uNAG and Cystatin C are useful biomarkers of DN, and in monitoring disease progression. It is known that several factors other than DN can increase the secretion of some of these tubular markers; therefore, combining markers can increase the sensitivity and specificity of these markers.

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