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Study of urine aquaporine-5 as a biomarker for diabetic nephropathy

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Abstract
Background: Diabetic nephropathy (DN) is a significant and enduring consequence of diabetes, and it is the primary cause of end-stage renal disease (ESRD). The objective of this research was to investigate the capability of urine aquaporin-5 (AQP-5) as a diagnostic marker for DN.
Methods: This present research was done on 80 subjects aged over 25 years old, 20 apparently healthy volunteers and 60 patients with diabetes mellitus (DM). Subjectswere categorized into 2 groups: Group I: 20 apparently healthy individuals (9 males and 11females) as control group with an age ranged from 28 to 65 years. Group II: 60 patients with DM, were subdivided into equal groups: Group IIA: patients without nephropathy with their ages ranged from 26 to 70 years and Group IIB: patients with nephropathy with their ages ranged from 34 to 77 years.
Results: ROC curve between group-IIB and group (I+IIA) according to level of urine AQP5 (ng/mL) with Cut off value >1.2ng/mL, sensitivity = 53.33%, specificity = 94%, positive predictive value (PPV) =84.2%, negative predictive value (NPV) = 77% and area under curve (AUC) 0.744. ROC curve between group (I+IIA) and group-IIB, as regard urine AQP5/creatinine ratio (ng/g) with Cut off value >1095.7 ng/g, sensitivity = 63.33%, specificity = 90%, PPV=79.2%, NPV = 80.4% &AUC= 0.807.There were significant positive correlations of urine AQP5/creatinine ratio with duration of DM (r = 0.665, P-value <0.001), HbA1C% (r = 0.442,P-value =0.014), serum creatinine (r = 0.583, P-value = 0.001), BUN (r = 0.751, P-value <0.001), ACR (r = 0.672, P-value<0.001), and urine AQP5 (r = 0.814, P-value <0.001).
Conclusions: The urine AQP5/creatinine ratio may serve as a potential non-invasive biomarker for the diagnosis and prognosis of DN.

Keywords: AQP5, DN, DM, eGFR

Introduction

Diabetes Mellitus (DM) is a prevalent metabolic condition that affects more than 350 million individuals globally. The incidence of both type 1 and type 2DM in adults has been progressively rising over the last several decades [1].

DN is a consequential condition that arises from diabetes and is the leading factor behind ESRD. Medically, it is defined by the presence of chronic albuminuria and is often accompanied with a gradual decline in the glomerular filtration rate (GFR), the development of diabetic retinopathy and an increase in arterial blood pressure [2].

Albuminuria, the conventional glomerular biomarker for DN, is not sufficiently sensitive or specific for identifying the early stages of DN. In addition, some individuals with DN and ESRD may not have substantial levels of albuminuria [3], while tubular dysfunction in DN may begin even before the onset of microalbuminuria. Therefore, tubular markers are more effective in detecting DN, especially in the early stages when there is no presence of albumin in the urine (Normoalbuminuric stage) [4].

Water channels aquaporins; AQPs 1-4 and AQP7 are expressed in the normal renal tubules and are particularly important for body water balance [5].

AQP-5 plays a role in the generation of pulmonary secretions, saliva and tearsis not present in normal human kidneys [6].

In DN, there is downregulation of Dot1a encoded by Dot1l gene that targets the transcription of AQP5 in AQP2-expressing cells in the renal collecting ducts leading to upregulation of AQP5 in those cells. The abnormally upregulated AQP-5 in the tubular cells interacts with the normally present AQP2 in the perinuclear region, impairing its localization on the apical cell surface leading to polyuria and polyuria-induced kidney damage [7].
The aim of this work was to investigate urine AQP-5 as a biomarker for DN.

Patients and Methods
This research was conducted on a sample of 80 individuals, all of whom were above the age of 25. The sample consisted of both genders, with 20 being healthy volunteers and 60 being patients diagnosed with DM. The research was conducted with clearance from the Ethical Committee of Tanta University Hospitals in Tanta, Egypt. The approval code is 31976/12/17. The patient provided a well-informed written consent.
The exclusion criteria included pregnancy, primary glomerulonephritis or any other renal-damaging ailment, urinary tract infection, severe heart failure, and the presence of other major medical problems such as cancer.

The participants were categorized into 2 groups: Group I (control group) consisted of 20 healthy adults, including11 females and 9 men. The age of the participants varied from 28 to 65 years. Group II consisted of 60 patients with DM, who were split into equal subgroups: Group IIA consisted of patients without nephropathy, aged between 26 and 70 years. Group IIB consisted of patients with nephropathy, aged between 34 and 77 years.

Among the laboratory tests that were performed were the following: complete blood count (CBC), postprandial blood sugar measurements (measured 2 hours after eating), haemoglobin A1C, liver and kidney functions, ESR (red blood cell settling rate), urine analysis, albumin to creatinine ratio (ACR), estimated glomerular filtration rate (eGFR), and AQP-5level measurement using the Enzyme linked immunosorbent assay (ELISA) technique. In order to ensure that all patients were in good health, we took the time to review their medical histories, conduct clinical examinations (which included a thorough examination of the patient's eyes), and measure their blood pressure and cardiac status. Echocardiography and other radiological tests were part of the laboratory investigations.

Urine sampling: The first morning urine sample was aseptically obtained from each participant, immediately voided into a sterile container, and then centrifuged within 2 hours after collection at a speed of 1000 x g for 20 minutes to eliminate any solid particles. The liquid portion was then partitioned in the following manner: A volume of about 2 mL was held for ELISA at a temperature of -20 degrees Celsius or below, for a maximum duration of two months, in order to prevent any degradation of bioactivity and minimize the risk of contamination. To prevent repeated freeze-thaw cycles, about 3 mL of the sample was submitted to the laboratory for calculation of urinary ACR.

Blood sampling: 5 millilitres of peripheral venous blood was collected from most subjects by the use of disposable sterile plastic syringe under complete aseptic technique. The sample was fractionated as follows: 2 mL of blood was delivered into a tube containing 50 µl of EDTA (10% conc.) for HbA1c test and 3 mL of blood was taken into an empty plastic tube and left to clot or centrifugated. The serum was then separated for routine investigations.

Detection of urineAQP-5 by human AQP-5 ELISA kit
Catalog No: E0583h, supplied by Wuhan EI Aab Science Co., China. This immunoassay kit allows accurate quantification of human AQP-5concentrations in serum, plasma, urine, tissue homogenates, cell culture super nates, and other biological fluids. The microtiter plate included in this kit is pre-coated with an antibody that selectively binds to AQP-5. Subsequently, the suitable wells of the microtiter plate are supplemented with standards or samples, along with a biotin-conjugated polyclonal antibody preparation that selectively binds to AQP-5. Afterward, every well of the microplate is exposed to Avidin that is linked to Horseradish Peroxidase (HRP) and allowed to undergo incubation.
Afterwards, A TMB substrate solution is added to each well. Colour alteration will only happen in wells that contain AQP-5, biotin-conjugated antibody, and enzyme-conjugated Avidin. In order to cease the enzyme-substrate reaction, a solution of sulphuric acid is injected. The subsequent change in colour is then measured using spectrophotometry at a precise wavelength of 450 nm ± 2 nm to determine its quantity. The quantification of AQP-5 in the samples is determined by comparing the optical density (O.D.) of the samples to the standard curve.

Wash Buffer: A total volume of 750 mL of Wash Buffer was obtained by diluting 30 mL of Wash Buffer Concentrate with deionized or distilled water.

Standard: A stock solution was generated by diluting the Standard with 1.0 mL of Sample Diluent, resulting in a concentration of 10.0 ng/mL. Prior to completing subsequent dilutions, the standard was left undisturbed for at least 15 minutes, with moderate stirring. A concentration of 10.0 ng/mL was used for the pure standard as a reference point. The Sample Diluent functioned as the standard reference at a concentration of 0 ng/mL.

Detection Reagent A and B: With the use of Assay Diluent A (1:100) and Assay Diluent B (1:200), they were each diluted to a working concentration**.**

Detection Range: 0.156-10 ng/mL.

Sensitivity: less than 0.074 ng/mL

Estimation of urine ACR
ACR was estimated by the following two steps.

Estimation of urine albumin by microalbumin reagent kit: The manufacturer's location is located at 250 S. Kraemer Blvd. Brea, CA 92821, USA. The firm responsible is Beckman Coulter, Inc. The phenomenon of light scattering in a solution is closely correlated with the dimensions, configuration, and abundance of the immune complexes formed. Turbidimeters quantify the decrease in incoming light resulting from reflection, absorption, or scattering. The Beckman Coulter technique relies on measuring the reduction in light transmission (increase in

absorbance) caused by the formation of complexes during the antigen-antibody response in a solution containing suspended particles.

Estimation of urine creatinine by creatinine reagent kit
Produced by Beckman Coulter, Inc., Brea, CA 92821 USA, 250 S. Kraemer Blvd. In this creatinine technique, which is a kinetic variation of the Jaffe procedure, picric acid forms a yellow-orange combination with creatinine at alkaline pH.

Estimation of urine ACR
ACR was estimated by using albumin and creatinine concentrations established in urine samples through this formula:

$$ACR = \frac{\text{Albumin (mg/dL)}}{\text{Creatinine (g/dL)}} = \frac{\text{mg Albumin}}{\text{g Creatinine}} = \frac{\text{Excreted Albumin (mg)}}{\text{24hr}}$$

Creatinine concentration in mg/dl was converted to g/dl by division on 1000. Normal ACR: less than 30 mg/g.

Estimation of glomerular filtration (eGFR)
The GFR was estimated based on the MDRD equation as follows: GFR (ml/min/1.73 m2) = 186 x [serum creatinine (mg/dl)-1.154 x age (ys)-0.203 x 0.742 if female].

Statistical analysis
The statistical analysis was performed using SPSS v27 (IBM©, Chicago, IL, USA). The normality of the data distribution was evaluated by doing the Shapiro-Wilks test and examining histograms. The numerical data were presented as the mean and standard deviation (SD) and were evaluated using an analysis of variance (ANOVA) test, followed by a post hoc test (Tukey). The median and interquartile range (IQR) were used to present the quantitative non-parametric data. The data were compared between each group using the Kruskal-Wallis test, followed by the application of the Mann Whitney-test. The qualitative variables were measured in terms of frequency and percentage (%) and assessed using the Chi-square test. The Pearson correlation coefficient (r) was calculated to ascertain the strength and direction of the association between two continuous numerical variables, with at least one of them adhering to a normal distribution. An evaluation of the test's characteristics was conducted using a receiver operating characteristic (ROC) curve, which determined the best threshold value and the area under the curve (AUC). A two-tailed P value of 0.05 or less was considered statistically significant.

Results
Group-IIB saw a substantial increase in the duration of DM in comparison to group-IIA. There was no significant difference in terms of sex between group-I, group-IIA, and group-IIB, as shown by a non-statistically significant P-value of 0.992. Table 1.

Table 1: Comparison between of sex distribution and duration DM in all studied groups

		Groups			P-value
		Group IIA	Group IIB		
Duration of DM (Years)		4.63±2.31	12.83± 6.07		<0.001*
		Group I	Group IIA	Group IIB	
Sex	Male	9(45%)	14(46.67%)	14(46.67%)	0.992
	Female	11(55%)	16(53.33%)	16(53.33%)	

Data are presented as mean ± SD or frequency (%), DM

There was no discernible disparity in HbA1C and FBS levels between group-IIB and group-IIA. Group-IIB and group-IIA exhibited a substantial rise when compared to group-I. In relation to fasting blood sugar (FBS) and glycated hemoglobin (HbA1C).There were no notable disparities seen between group-I and group-IIA in relation to serum creatinine level, BUN, eGFR, ACR, urine AQP5 level, and urine AQP5/creatinine ratio. Group-IIB demonstrated a significant elevation in serum creatinine and BUN levels in comparison to group-I and group-IIA, suggesting a remarkable increase. Group-IIB showed a notable decline in eGFR when compared to group-I and group-IIA, with a P-value of less than 0.001. Group-IIB had a significant rise in ACR, urine AQP5, and urine AQP5/creatinine ratio in comparison to group-I and group-IIA. Table 2.

Table 2: Comparison between laboratory parameters in all different studied groups

	Group I	Group IIA	Group IIB	P-value	TUKEY'S Test		
					I and IIA	I and IIB	IIA and IIB
FBS (mg/dL)	88±7.24	160.5±45.94	161.63±35.75	<0.001*	<0.001*	<0.001*	0.992
HbA1C	4.63±0.56	9.18±2.04	10.01±1.88	<0.001*	<0.001*	<0.001*	0.158
Serumcreatinine level (mg/dL)	0.75±0.11	0.79± 0.11	2.33±1.51	<0.001*	0.989	<0.001*	<0.001*
BUN(mg/dL)	12.49±3.66	14.04±4.11	28±13.28	<0.001*	0.813	<0.001*	<0.001*
eGFR(ml/min/1.73m^2)	107.67±8.84	100.97±12.83	44.12±25.77	<0.001*	0.414	<0.001*	<0.001*
	Specific laboratory parameters				Mann-Whitney Test		
ACR (mg/g)	8.25-6.78	12.30-12.20	527.50-2693	<0.001*	0.198	<0.001*	<0.001*
Urine AQP5 (ng/ml)	0.080- 0.080	0.174-0.833	1.265-1.783	<0.001*	0.058	<0.001*	0.010*
Urine AQP5/Creatinine(ng/g)	170.10-213.83	122.20-594.95	2672.85-4899.55	<0.001*	0.937	<0.001*	<0.001*

Data are presented as mean ± SD or median (IQR), FBS, BUN, eGFR, ACR, AQP5.

The eGFR-based staging of group-IIB was tabulated according to K/DOQI classification of CKD, showing that group IIB includes 14 (46.67%) cases related to stage II, 6 (20%) cases related to stage III and 10 (33.3%) cases related to stage V. Table 3

Table 3: eGFR-based staging of group-IIB (K/DOQI classification of CKD)

Stages	Number (%)	eGFR
Stage II	14 (46.67%)	60-89
Stage III	6 (20%)	30-59
Stage V	10 (33.3%)	<15 or dialysis

Data are presented as frequency (%), eGFR.

No statistically significant disparity was seen in the urine AQP5 level between stage 3 and stage 5 (P-value = 0.233). The median concentration of urine AQP5 in stage 3 and stage 5 was significantly higher than that in stage 2 (P-value = 0.001). The median urine AQP5/creatinine ratio in stage 3 was considerably higher than that in stage 2 (P-value = 0.003), and in stage 5 it was likewise significantly higher than that in stage 2.Table 4

Table 4: Comparison between group-IIB stages as regard urine AQP5/creatinine ratio and urine AQP5 level

	N		P-value	Mann-Whitney Test	
Urine AQP5 (ng/ml)					
Stage 2	14	0.105 (0.928)	<0.001*	2&3	0.001*
Stage 3	6	2.420 (2.053)		2&5	0.001*
Stage 5	10	1.715 (1.570)		3&5	0.233
Urine AQP5/Creatinine (ng/g)					
Stage 2	14	213.90 (1719.95)	<0.001*	2&3	0.003*
Stage 3	6	3691.95 (3113.58)		2&5	<0.001*
Stage 5	10	5606.30 (9020.35)		3&5	0.329

Data are presented as median (IQR), AQP 5.

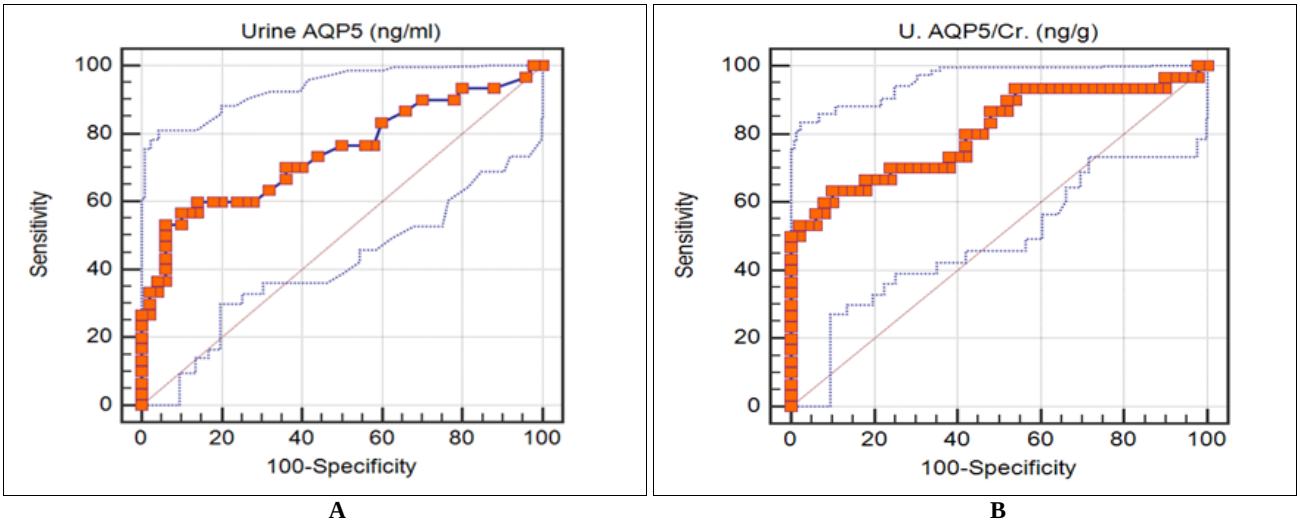
The urine AQP5/creatinine ratio showed significant positive correlations with the duration of DM (r = 0.665, P-value <0.001), HbA1C% (r = 0.442, P-value =0.014), serum creatinine (r = 0.583, P-value = 0.001), BUN (r = 0.751, P-value <0.001), ACR (r = 0.672, P-value<0.001), and urine AQP5 (r = 0.814, P-value <0.001).The urine AQP5/creatinine ratio showed a strong negative connection with eGFR (r = -0.642, P-value<0.001). The study found no statistically significant link between the urine AQP5/creatinine ratio and FBS level (r = 0.109, P-value = 0.565). Table 5

Table 5: Correlations of urine AQP5/creatinine ratio (ng/g) with different variables in group-IIB

U. AQP5/Cr. (ng/g)		
Group IIB		
	r	**P-value**
FBS (mg/dL)	0.109	0.565
HbA1C%	0.442	0.014*
Serum Cr. (mg/dL)	0.583	0.001*
BUN (mg/dL)	0.751	<0.001*
eGFR (ml/min/1.73m^2)	-0.642	<0.001*
ACR (mg/g)	0.672	<0.001*
Duration of DM (Years)	0.665	<0.001*
U. AQP5 (ng/mL)	0.814	<0.001*

r: Pearson correlation coefficient, FBS, BUN, eGFR, ACR, DM,AQP 5

ROC curve between group(I+IIA) and group-IIB as regard urine AQP5 level (ng/mL) with Cut off value >1.2ng/mL, sensitivity = 53.33%, specificity = 94%,PPV=84.2%, NPV = 77% and AUC= 0.744.ROC curve between group (I+IIA) and group-IIB as regard urine AQP5/creatinine ratio (ng/g) with Cut off value >1095.7 ng/g, sensitivity = 63.33%, specificity = 90%, PPV=79.2%, NPP = 80.4% &AUC= 0.807. Figure 1



A **B**

Fig 1: ROC curve between group (I+IIA) and group-IIB as regard A: urine AQP5 level (ng/mL), B: urine AQP5/creatinine ratio (ng/g)

Discussion

The leading cause of ESRD worldwide, DN is a common microvascular complication of diabetes that often requires dialysis and/or a kidney transplant. The hallmarks of DN include a slow but steady increase in albuminuria and a decline in GFR, the latter of which is often associated with hypertension [8]. Aqueduct AQP5 plays a vital role in the synthesis of saliva, tears, and pulmonary secretions [9] Immunoblotting analysis reveals that AQP5 is not detectable in normal mouse and human kidneys, suggesting that it has a little or minor role in normal kidney function [7].

Wu *et al.* [7] has shown that AQP5 is increased in kidney biopsies taken from individuals with DN, particularly in the cells of the inner medullary collecting duct (IMCD3). On that basis, there are two recent studies that used an AQP5-specific ELISA to investigate urine AQP5 as a diagnostic biomarker for DN [10, 11].

The current research demonstrated a statistically significant difference in the average duration of diabetes between the DN group and the DM group. Consistent with our findings, Lu *et al.* [10] and many other research [12, 14] observed a substantial correlation between the duration of diabetes and

nephropathy. Chronic hyperglycaemia is a major contributor to the development and advancement of DN [15].

In the present study, the average fasting blood sugar (FBS) level showed a substantial rise in both the DN and DM groups. However, there was no significant difference in FBS level between the DM group and the DN group. The results align with the statistical findings reported in Lu *et al*. [10] using FBS. Regarding the HbA1C %, this study demonstrated that there was no statistically significant difference between the DM and DN groups. The mean value of HbA1C % in both DM & DN groups was significantly increased compared that in the control group. These results agree with the statistical results of HbA1C% presented by Lu *et al*. [10]. In this work, the elevated HbA1c% and fasting blood glucose levels found in both the DM and DN groups suggest poor glycaemic control. This finding is consistent with earlier studies that have identified insufficient glycaemic control as a significant risk factor for the development and progression of DN [13, 14, 16, 18]. Regarding serum creatinine levels, this research found no significant difference between the control group and the group with DM. However, the mean serum creatinine level in the group with DN was considerably higher compared to both the control and DM groups. These results are in accordance with the serum creatinine statistical results demonstrated by Lu *et al*. [10] as regard BUN level, this study revealed that the control group and DM group did not show any significant difference. However, the BUN mean level in the DN group was considerably higher compared to both the control and DM groups. These results align with the BUN statistical findings presented by Lu *et al*. [10].

Measuring BUN and serum creatinine is the most basic method to assess renal function. In the context of renal illness, these chemicals experience abnormal excretion, leading to their storage inside the body and subsequent elevation of their blood levels [19]. In this work, the serum creatinine and BUN results in the DN group go along with this fact. In this work, the main laboratory tests used to diagnose the cases of DN were urinary albumin excretion (UAE), expressed as UACR (mg/g) & eGFR (ml/min/1.73m^2) to be consistent with the definition of DN. DN is often characterized by an increase in urinary albumin excretion (UAE) and a decline in kidney function, as shown by elevated plasma creatinine levels, lower estimated creatinine clearance, or diminished GFR [20]. Therefore, the statistical results of eGFR and UACR that are related to the DN group were significant compared to the other two groups.

In the current study, there was no significant difference between the control group & DM group as regard eGFR level while the mean value of eGFR in the DN group was significantly decreased compared to that in both control & DM groups. These results are agreed with the eGFR statistical results demonstrated by Lu *et al*. [10] and Rossi *et al*. [11] Gao and Zhang [21] also suggested that Urinary AQP5, a potential indicator of tubular dysfunction, is autonomously linked to the reduction of eGFR in individuals diagnosed with type 2 diabetes and nephropathy. The eGFR decline in DN is mainly attributed to chronic hyperglycaemia that affects Mesangial cells play a crucial role in preserving the shape of glomerular capillaries and regulating glomerular filtration via smooth muscle activity [22].

As regard UACR, this study revealed that there was no significant difference between the control & DM group while it was significantly increased in the DN group compared to the other two groups. These results are agreed with UACR statistical results demonstrated by Lu *et al*. [10] and Rossi *et al*. [11].

This study observed that the urine AQP5 level did not show a significant disparity between the control group and the group with diabetes. However, it was notably elevated in the group with DN when compared to both the control group and the group with diabetes. These findings are consistent with the studies undertaken by Lu *et al*. [10], which produced comparable statistical results. Furthermore, these findings align with the research done by Wu *et al*. [7], which demonstrated an increased expression of AQP5 in the collecting ducts of individuals with DN.

As regard urine AQP5/creatinine ratio, this research revealed that there was no statistically significant difference between the control and DM group, however it was notably greater in the DN group compared to the other two groups. This finding corresponds to the studies conducted by Lu *et al*. [10] and Rossi *et al*. [11], which proposed that urine AQP5/creatinine, might serve as a diagnostic biomarker for DN. In this study, the DN group was classified into stages II, III &V based on the eGFR values in the K/DOQI classification of CKD [23]. DN Stages II, III & V were coinciding with microalbuminuria, macroalbuminuria & massive albuminuria ESRD respectively [24].

As regard urine AQP5 level, this study found that there was no significant difference between stages III & V of DN while it was significantly higher in stages III & V than in stage II. Therefore, urine AQP5 level will be significantly higher in the macroalbuminuric stages (stages III & V) than in the macroalbuminuric stage (stage II), indicating its possible role in the DN progression. This statistical finding contradicts the study conducted by Lu *et al*. [10], which reported no significant variation in urine absolute AQP5 content throughout different phases. The possible pathological role of AQP5 in the DN progression has been supported by Wu *et al*. [7] found that the abnormally upregulated AQP-5 in the tubular cells of DN patients interacts with the normally present AQP2 in the perinuclear region, impairing its localization on the apical cell surface leading to polyuria and polyuria-induced kidney damage.

In this study, the similar statistical results of urine AQP5 level and urine AQP5/creatinine ratio as regard stages of DN is most possibly attributed to the highly significant positive correlation between these two variables.Lu *et al*. [10] focused on urine AQP5/creatinine (ng/g) rather than the absolute urine AQP5 concentration (ng/ml) or total excretion of AQP5 (ng/24h) as it yielded the highest area under the ROC curve. For the same reason, this study also focused on urine AQP5/creatinine ratio.

The current research also has shown a substantial and positive correlation between the ratio of urine AQP5 to creatine and serum creatinine, BUN, and ACR. Additionally, there was a significant and negative correlation between this ratio and eGFR. These findings align with the results reported by Lu *et al*. [10], suggesting that the ratio of urine AQP5 to creatinine might serve as a potential new biomarker for diagnosing and predicting the progression of DN. In this study, the ROC analysis was performed after combining normal controls and DM patients as the “non-DN” group. When the ROC analysis done by using urine AQP5/creatinine ratio, it showed a sensitivity of 63.3% & a specificity of 90% for the diagnosis of DN, whereas the ROC analysis done by using urine absolute AQP5 concentration showed a sensitivity of 53.3% & a

specificity of 94%. The ROC area of urine AQP5/creatinine was 0.807 while that of urine AQP5 concentration was 0.744; Therefore, urine AQP5/creatinine may facilitate the differentiation of DN from normal and DM subjects. These findings are consistent with Lu *et al*. [10].

Our research suggested that our results should be corroborated through more extensive and longitudinal investigations. Subsequent investigations employing sizable cohorts comprising adolescents, young adults, and individuals with type 1 diabetes are necessary to validate our conclusions. Establishing the early detection of AQP5 in the urine of patients exhibiting subclinical indicators of DN would also be facilitated by conducting additional research on a larger cohort of patients, which would include individuals with grade I DN

Conclusion: The urine AQP5/creatinine ratio may serve as a potential non-invasive biomarker for the diagnosis and prognosis of DN.

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Conflict of Interest: Nil

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