



## **Study of Urinary Level of Vitamin D Binding Protein in Diabetic Nephropathy**

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### **Authors' contributions**

*This work was carried out in collaboration among all authors. All authors read and approved the final manuscript.*

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## **ABSTRACT**

**Background:** Diabetic nephropathy is usually a clinical diagnosis made based on the presence of albuminuria and or reduced estimated glomerular filtration rate in the absence of signs or symptoms of other primary causes of kidney damage. The aim of this work was to study urinary level of vitamin D binding protein as an early predictor for DN and its role in early diagnosis of diabetic nephropathy.

**Methods:** This prospective cohort observational study was carried out on 60 subjects. All subjects were divided into 3 equal groups: Group I: Patient with type 2 DM. with UACR<30 mg/gm. Group II: Patient with type 2 DM. with UACR 30-300mg/gm. Group III: Healthy Controls (matched with other groups as regard the age, gender and body mass index). All patients were subjected to routine laboratory investigation and specific laboratory investigation (Urinary Vitamin D Binding Protein by ELISA).

**Results:** The area under the ROC curve of urine VDBP levels for the diagnosis of DN was 90%. The analysis rendered an optimum cut-off value of  $\geq 125\mu\text{g/ml/mg}$  corresponding to 77.50% sensitivity and 85.00% specificity. There was significant positive correlation between Urinary VDBP level and FBS serum level (P value<0.001). There was significant positive correlation between Urinary VDBP level and HA1C serum level (P value<0.001)

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**Conclusions:** Our findings indicate that UVDBP levels may be a potential biomarker for early detection of DN. In the present study, urine samples were collected from patients with DN only but not from patients with additional nephropathies. This may have caused an overestimation of the specificity of VDBP as a biomarker for the detection of DN.

*Keywords: Urinary level; vitamin D binding protein; diabetic nephropathy.*

## 1. INTRODUCTION

The term diabetes means "passing through," referring to polyuria, a symptom that has historically been prevalent in persons with diabetes. The presence of decreased renal function or higher urine albumin excretion (UAE) or both characterizes diabetic nephropathy [1,2].

Approximately half of the patients with type 2 DM will have evidence of chronic kidney disease [3].

"Diabetic nephropathy is often diagnosed clinically based on the presence of albuminuria and/or a decreased estimated glomerular filtration rate in the absence of other major causes of kidney disease" [4].

"Urinary albumin excretion is the current gold standard for the detection and monitoring of nephropathy and cardiovascular risk in diabetic patients; however, its predictive power is limited, and research is focusing on biomarkers that may offer greater sensitivity and earlier detection to facilitate earlier intervention" [5].

Several serum circulating biomarkers may also help to identify those who will develop nephropathy in patients with DM to those patients at risk to progress to end stage kidney disease [6].

"Vitamin D binding protein (DBP), formerly known as Group-specific component (Gc-globulin), is a 51–58kDa multifunctional serum glycoprotein synthesized in large amounts by hepatic parenchymal cells and secreted into the circulation as a monomeric mature peptide of 458 residues and three structural domains" [7].

The frequency of 25-vitamin D insufficiency increases as CKD progresses and exceeds 80 percent in individuals with stage 5 CKD [8].

Although repletion with high-dose ergocalciferol (20,000 units/week × 9 months) is regarded safe, only around half of haemodialysis patients attain the target level [9]. The purpose of this study was to examine the urinary level of vitamin D binding

protein as an early predictor of DN and its function in the early diagnosis of diabetic nephropathy.

## 2. PATIENTS AND METHODS

This prospective cohort observational study was carried out on 60 subjects 40 patients with type 2 diabetes and 20 subjects apparent healthy matched with other groups as regard the age, gender. This study was conducted from 2018-2019 at Clinical Pathology and Internal Medicine Department Tanta University Hospitals (Nephrology Unit), Faculty of Medicine, Tanta University in Tanta.

Patient with type 1 diabetes mellitus, collagen diseases as rheumatoid arthritis and SLE, cancer, chronic liver disease, pregnancy, other causes of kidney diseases rather than D.M and aged less than 65 years old were excluded.

All subjects were divided into 3 groups: Group I: Patient with type 2 DM. with UACR<30 mg\gm (20 patients). Group II: Patient with type 2 DM. with UACR 30-300mg\gm (20 patients). Group III: Healthy Controls (20 controls) (matched with other groups as regard the gender, age and body mass index).

All patients were subjected to: Clinical examination, complete history taking, routine laboratory investigation (Fasting and 2 hours post prandial blood sugar (mg/dl), serum creatinine, urinary albumin creatinine ratio (mg/gm) and glycated Hb A1c) and specific laboratory investigation (Urinary Vitamin D Binding Protein by ELISA).

Blood sample was taken as 7 ml of venous blood were collected from each subject in two samples 5ml fasting and 2ml post prandial after his consent by the use of disposable sterile plastic syringe.

Nearly 20ml of clean-catch midstream urine samples were collected in a sterile plastic tube and centrifuged for 10 minutes at 3000rpm, 4°C. The supernatant was frozen at -80°C for further

examination. Repeated freeze-thaw cycles were avoided. Spot urinary albumin and creatinine concentrations were calculated and presented as the urinary albumin (mg)/creatinine (gm) ratio (UACR).

**Blood glucose level:** "It is measured spectrophotometrically, the absorbance to be read between 505 nm to 550 nm. Glucose oxidase (GOD) catalyses the oxidation of glucose to gluconic acid. The formed hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>), is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD). The intensity of the color produced is related to the sample's glucose content" [10].

**Serum creatinine:** "It is measured spectrophotometrically at 520 nm. The jaffe reaction for measuring creatinine is primarily based on the reaction of creatinine with picrate ion in an alkaline media to yield an orange red complex, the intensity of that colour is directly proportional to the concentration" [11].

**Urinary ACR using morning urine sample:** It is measured spectrophotometrically, read at 520 nm on the Model 200-N spectrophotometer. Quantitative determination of urine microalbumin was done using Fineware™ microalbumin rapid quantitative test using Fineware™ fluorescence immunoassay meter. UACR was determined by dividing albumin (mg) by creatinine (gm) [12].

**HbA1c.:** "It is measured by Twin A1c Analyzer. This method uses the interaction of antibody and antigen to determine the HbA1c in whole EDTA blood. HbA1c in test samples is absorbed onto latex particles surface, which react with Anti-HbA1c (antigen-antibody reaction) and leads to agglutination. The amount of agglutination is expressed as absorbance. The HbA1c value is obtained from a calibration curve" [13].

### 2.1 Determination of Urine Vitamin D Binding Protein

Urine Vitamin D Binding Protein was determined by Omnikine human TM a sandwich enzyme-linked immunosorbent assay manufactured by Sun Red company (catalogue number 201-12-1403). This test utilizes the sandwich enzyme immunoassay method to determine the concentration of Human Vitamin D-binding protein (DBP) in samples. Vitamin D-binding protein (DBP) was added to monoclonal antibody Enzyme well which is pre-coated with Human Vitamin DBP monoclonal antibody incubation, DBP antibodies were labelled with biotin, and combined with Streptavidin-HRP to form immune complex; We repeated incubation and washing to eliminate the uncombined enzyme. Then we added chromogen solution A, B, the colour finally becomes yellow. The correlation between chroma of colour and concentration of Human Substance DBP was positive.

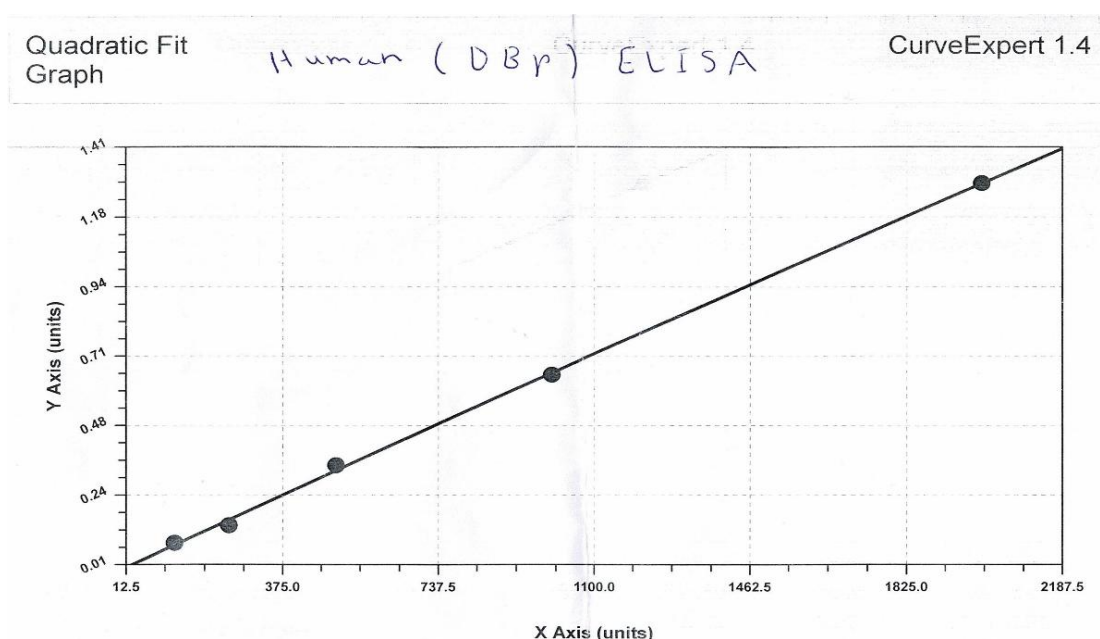


Fig. 1. Results of the level of urinary DBP in the samples by ELISA test

**Table 1. Materials supplied in the test kit**

1	Standard (4000µg/ml)	0.5ml
2	Standard diluent	3ml
3	Micro- Elisa Strip plate	12wellx8strips
4	Str-HRP-Conjugate Reagent	6ml
5	30xwash solution	20ml
6	Biotin-DBP Ab	1ml
7	Chromogen solution A	6ml
8	Chromogen solution B	6ml
9	Stop solution	6ml
10	Instruction	1
11	Closure plate membrane	2
12	Sealed bags	1

**2.2 Statistical Analysis**

SPSS v26 was used to do statistical analysis (IBM Inc., Chicago, IL, USA). Using the ANOVA (F) test, quantitative variables were given as mean and standard deviation (SD) and compared between the two groups. The Chi-square test was utilized to analyze qualitative variables expressed as frequency and percentage (percent). A two-tailed P value less than or equal to 0.05 was deemed statistically significant.

**3. RESULTS**

Regarding sex distribution, there were no statistically significant differences between the three groups (P value=0.150). Regarding age distribution, there were statistically significant

differences between the three groups (P value<0.001) Table 2.

Regarding duration of D.M., there were no statistically significant differences between the three groups (p-value=0.880) Table 3.

Regarding level of creatinine, there were statistically significant differences between the three groups (P value<0.001). Regarding level of albumin creatinine ratio, there were statistically significant differences between the three groups (P value<0.001). Regarding level of albumin creatinine ratio, there were statistically significant differences between the three groups (P value<0.001). Regarding level of Haemoglobin A1C, there were statistically significant differences between the three groups as

**Table 2. Sex and age distribution in studied groups**

	Groups						P-value
	Group I		Group II		Group III		
	N	%	N	%	N	%	
Sex							
Male	14	70.00	12	60.00	8	40.00	0.150
Female	6	30.00	8	40.00	12	60.00	
Total	20	100.00	20	100.00	20	100.00	
Age (Years)							
Mean ± SD	51.150 ± 8.222		47.350 ± 7.896		39.400 ± 4.500		<0.001*
Range	33-61		33-60		33-50		P1 0.215 P2 <0.001* P3 0.002*

\*: Significant as P value ≤ 0.05, P1: P value between group I & II, P2: P value between group I & III, P3: P value between group II & III

**Table 3. Duration of Diabetes Mellitus (DM) between group I and group II**

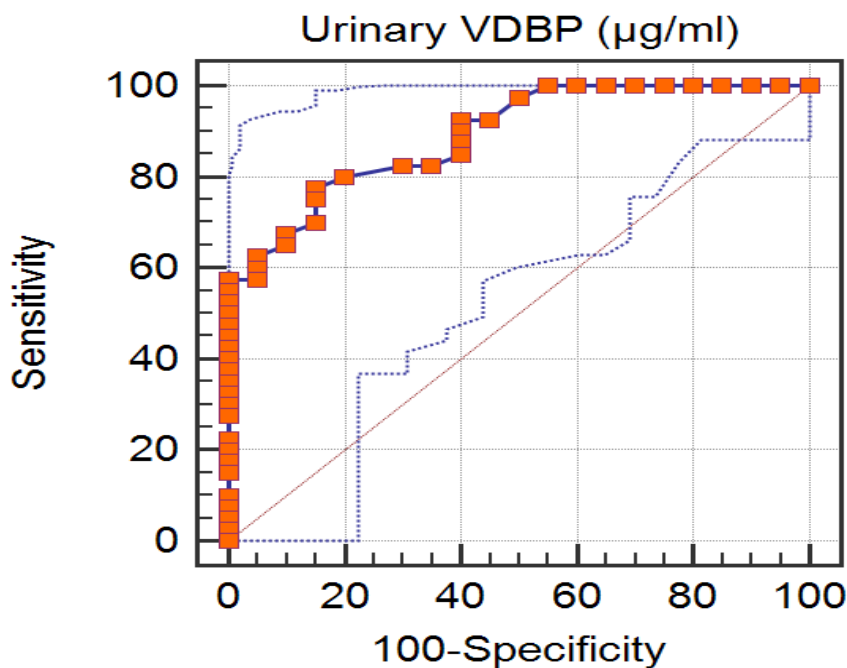
Duration of DM (Years)	Group I	Group II	P-value
Mean ± SD	5.900 ± 2.269	3 ± 3.804	0.880
Range	3-10	3-20	

DM: Diabetes mellitus

**Table 4. The level of creatinine (Cr) in studied groups**

		<b>Group I</b>	<b>Group II</b>	<b>Group III</b>	<b>P-value</b>
<b>Cr (mg/dl)</b>	<b>Mean ± SD</b>	1.079 ± 0.162	1.511 ± 0.067	0.995± 0.193	<b>&lt;0.001*</b>
	<b>Range</b>	0.8 - 1.34	1.4 - 1.6	0.7 - 1.3	P 1 <b>&lt;0.001*</b> P 2 0.191 P 3 <b>&lt;0.001*</b>
<b>ACR (mg/gm)</b>	<b>Mean ± SD</b>	17.450± .236	110.000 ± 46.793	12.800 ± 2.285	<b>&lt;0.001*</b>
	<b>Range</b>	10 - 25	45 - 200	8 - 17	P 1 <b>&lt;0.001*</b> P 2 0.851 P 3 <b>&lt;0.001*</b>
<b>FBS (mg/dl)</b>	<b>Mean ± SD</b>	109.000± 6.609	136.550 ± 11.436	81.000 ± 3.685	<b>&lt;0.001*</b>
	<b>Range</b>	98-123	118- 156	74-90	P 1 <b>&lt;0.001*</b> P 2 <b>&lt;0.001*</b> P 3 <b>&lt;0.001*</b>
<b>HA1C %</b>	<b>Mean ± SD</b>	6.720± 0.099	7.300± 0.173	5.205 ± 0.147	<b>&lt;0.001*</b>
	<b>Range</b>	6.5-6.9	7.1-7.8	5-5.5	P 1 <b>&lt;0.001*</b> P 2 <b>&lt;0.001*</b> P 3 <b>&lt;0.001*</b>
<b>Urinary VDBP (µg/ml)</b>	<b>Mean ± SD</b>	136.20 ± 22.291	225.100 ± 33.762	107.500± 26.904	<b>&lt;0.001*</b>
	<b>Range</b>	111-182	172-300	62-160	P 1 <b>&lt;0.001*</b> P 2 0.006* P 3 <b>&lt;0.001*</b>

\*: Significant as P value ≤ 0.05, P1: P value between group I& II, P2: P value between group I& III, P3: P value between group II& III. Cr: Creatinine, ACR: Albumin creatinine ratio, FBS: Fasting blood sugar, HA1C: Hemoglobin A1C, VDBP: Vitamin D Binding Protein



**Fig. 2. ROC curve of Urinary VDBP between group I and II and group III (healthy group)**

**Table 5. Correlation between Urinary VDBP and different studied parameters in group I (D.N1) and group II (D.N2)**

Correlations	Urinary VDBP ( $\mu\text{g/ml}$ )	
	r	P-value
Age (Years)	-0.162	0.317
ACR (mg/gm)	0.710	<0.001*
FBS (mg/dl)	0.771	<0.001*
HA1C %	0.783	<0.001*
Cr (mg/dl)	0.745	<0.001*
Duration of DM (Years)	-0.120	0.462

\*: Significant as P value  $\leq 0.05$ , Cr: Creatinine, ACR: Albumin creatinine ratio, FBS: Fasting blood sugar, HA1C: Hemoglobin A1C, VDBP: Vitamin D Binding Protein

(P value <0.001). There was statistically significant increase in level of Urinary VDBP in between groups (P value<0.001) Table 4.

There was significant positive correlation between urinary VDBP level and ACR, FBS, HA1C and Cr serum level (P value<0.001) Table 5.

For the diagnosis of DN the area under the ROC curve of urine VDBP levels was 90%. The analysis rendered an optimal cut-off value of  $\geq 125\mu\text{g/ml/mg}$  which corresponds to 77.50% sensitivity and 85.00% specificity Fig. 2.

#### 4. DISCUSSION AND CONCLUSION

“In 2007, around 246 million people worldwide had diabetes, a number that is expected to rise to 550 million by 2030” [14].

VDBP is a 58-kDa glycoprotein with a serum concentration between 300 and 600 mg/ml [15].

Clinically, a study by Zoidakis and colleagues [16] observed a significant decrease in VDBP levels in the urine of individuals with invasive bladder cancer, this is in accordance with the results obtained by Li and colleagues [17]. Moreover, Li and colleagues [17] also revealed that the expression levels of UVDBP were positively linked with bladder cancer pathology categorization. UVDBP may be a viable non-invasive biomarker for the early detection and successful monitoring of bladder cancer, according to their findings.

Concerning UVDBP our results were in agreement with those of Mirkoviae et al., [18] indicated that VDBP excretion in the urine may be a new biomarker of tubulointerstitial injury. They also indicated that damaged tubular epithelial cells in areas of tubulointerstitial fibrosis

may no longer be able to handle VDBP, resulting in gross VDBP loss into the urine, and that it can be modulated by antiproteinuric treatment in patients. Although the combination of the renin-angiotensin-aldosterone system blockade and dietary sodium restriction, an intervention considered optimal for renoprotection, considerably reduced VDBP excretion, they demonstrated that UVDBP excretion is increased early after renal injury and is associated with tubulointerstitial inflammation and fibrosis independently of albuminuria.

Concerning UVDBP our results were in agreement with those of Tian and colleagues, [19] who studied 105 Chinese individuals with diabetes and 45 healthy volunteers. The patients were divided into three groups according to the value of ACR: the DM group without nephropathy and albuminuria; the early DN group with microalbuminuria; and the overt DN group with macroalbuminuria. They demonstrated that UVDBP levels were significantly elevated in patients with DN compared with patients without nephropathy. Moreover, strong positive correlation was observed between expression levels of UVDBP and the parameters of kidney dysfunction. The analysis of their result rendered an optimum cut-off value of 552.243 ng/mg corresponding to 92.86% sensitivity and 85% specificity. They concluded that UVDBP levels are a potential biomarker for early detection and prevention of DN.

Concerning UVDBP our results were in agreement with those of Khodeir et al., [20] who studied “45 patients with type 2 diabetes mellitus and were classified into three groups (normoalbuminuric, microalbuminuric, and macroalbuminuric). Fifteen healthy participants served as the control group. The excretion levels of UVDBP were quantified with enzyme-linked

immunosorbent assay. The results showed that UVDBP levels were significantly elevated in patients of the DN3 and DN4 groups compared with those of the DN2 group and normal controls. Receiver operating characteristic analysis rendered that an optimum cut-off value of UVDBP corresponding to 90.0% sensitivity and 76.7% specificity is appropriate for detecting DN”.

Concerning UVDBP our results were in agreement with those of Fawzy and Abu AISel, [21] who studied “one hundred and twenty diabetic patients (DM type 2) and 40 age and sex-matched apparently healthy controls have been enrolled in the current preliminary case-control study. Early morning serum and urine samples have been obtained on the same day from all participants. Patients who were attending the Prince Hospital Outpatient Diabetic Clinics, Northern Borders Area, Saudi Arabia, were divided into 3 patient groups: (1) normoalbuminuria group (urinary albumin excretion rate < 30 µg/mg), (2) microalbuminuria group (at least two of three consecutive urine samples with albumin excretion rate 30–300µg/mg), and (3) macroalbuminuria group (albumin excretion rate > 300 µg/mg), (n = 40 per group)”. They demonstrated that UVDBP levels were significantly elevated in patients with DN compared with patients without nephropathy. The analysis of their result rendered an optimum cut-off value of 214.00 ng/ml/mg corresponding to 82.5% sensitivity and 65.0% specificity in Controls versus microalbuminuria diabetic patients and an optimum cut-off value of 216.00 ng/ml/mg corresponding to 98.8% sensitivity and 80.0% specificity in Normoalbuminuria group versus microalbuminuria diabetic patients. They concluded that: the current results suggested that uVDBP could be implicated in combination with other conventional biomarkers for the early prediction of DN.

Concerning parameters of kidney function our results revealed that urinary VDBP correlated positively with creatinine, HA1C, FBS, ACR and These results were in agreement with those of Tian and colleagues [19] and Khodeir et al. [20], Fawzy and Abu AISel [21], negatively with, Age were in agreement with Fawzy and Abu AISel [21].

“Concerning the analysis of our result of an optimum cut-off ,our results rendered an optimum cut-off value of  $\geq 125\mu\text{g/ml/mg}$  corresponding to 77.50% sensitivity and 85.00% specificity, were in agreement with those of Tian and colleagues”, [19] “the analysis of their result

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Limitations: An important limitation of the present study regarding the specificity of this biomarker should be considered when UVDBP detection is used for early prevention of DN. It has been demonstrated that UVDBP levels are closely associated with renal dysfunction. In the present study, urine samples were collected from patients with DN only but not from patients with additional nephropathies. This may have caused an overestimation of the specificity of VDBP as a biomarker for the detection of DN. Therefore, further studies including a larger sample and analyses of patients with various types of non-DN are required to clarify this issue.

## 5. CONCLUSIONS

Our findings indicate that UVDBP levels may be a potential biomarker for early detection of DN, but an important limitation in terms of the specificity of this biomarker should be considered when UVDBP detection is used for early prevention of DN. In the present study, urine samples were collected from patients with DN only but not from patients with additional nephropathies. This may have caused an overestimation of the specificity of VDBP as a biomarker for the detection of DN.

## ETHICAL APPROVAL AND CONSENT

A signed informed consent was obtained from the patient or relatives of the patients. The study was done after approval from the Ethical Committee Tanta University Hospitals.

## COMPETING INTERESTS

Authors have declared that no competing interests exist.

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