UNDER PEER REVIEW

Original Research Article

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

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 ≥ 125μg/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)

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.

Introduction:

The word diabetes means "passing through", referring to the polyuria, a symptom historically present in those affected by the disease. Diabetic nephropathy is defined by presence of impaired renal function or elevated urinary albumin excretion (UAE), or both [1, 2].

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

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 [4].

Urinary albumin excretion is currently the gold standard for detection and monitoring nephropathy and cardiovascular risk in patients with diabetes, however its predictive powers have limitations and research is focusing on biomarkers which 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), originally known as the Group-specific component (Gc-globulin), is a 51–58kDa multifunctional serum glycoprotein synthesized in large quantities by hepatic parenchymal cells and secreted into the circulation as a monomeric mature peptide of 458 residues and three structural domains [7].

The prevalence of 25-vitamin D deficiency increases with progression of CKD and approaches 80% in stage 5 CKD patients [8].

Although repletion with high-dose ergocalciferol (20,000 units/week × 9 months) is considered safe, it achieves the desired level in only about 50% of haemodialysis patients [9]. The aim of

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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.

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.

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

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 age, gender and body mass index).

All patients were subjected to: Complete history taking, clinical examination, 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.

Urine sample was taken as a clean-catch midstream urine samples (nearly 20ml) were collected into a sterile plastic tube and then centrifuged for 10 minutes at 3000rpm, 4°C. The supernatant was stored at −80°C for further analysis. Repeated freeze-thaw cycles were avoided. Spot urinary albumin and creatinine concentrations were measured and expressed 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_2O_2), is detected by a chromogenic oxygen acceptor, phenol, 4 – aminophenazone (4-AP) in the presence of peroxidase (POD). The intensity of the colour formed is proportional to the glucose concentration in the sample [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 Finecare ™ microalbumin rapid quantitative test using Finecare ™ fluorescence immunoassay meter. UACR was determined by dividing albumin (mg) by creatinine (gm) [12].

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

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 assay employs the quantitative sandwich enzyme immunoassay technique to assay the level 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 carried out incubation and washing again to remove the uncombined enzyme. Then we added chromogen solution A, B, the colour finally becomes yellow. The chroma of colour and concentration of Human Substance DBP of sample were positively correlated.

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

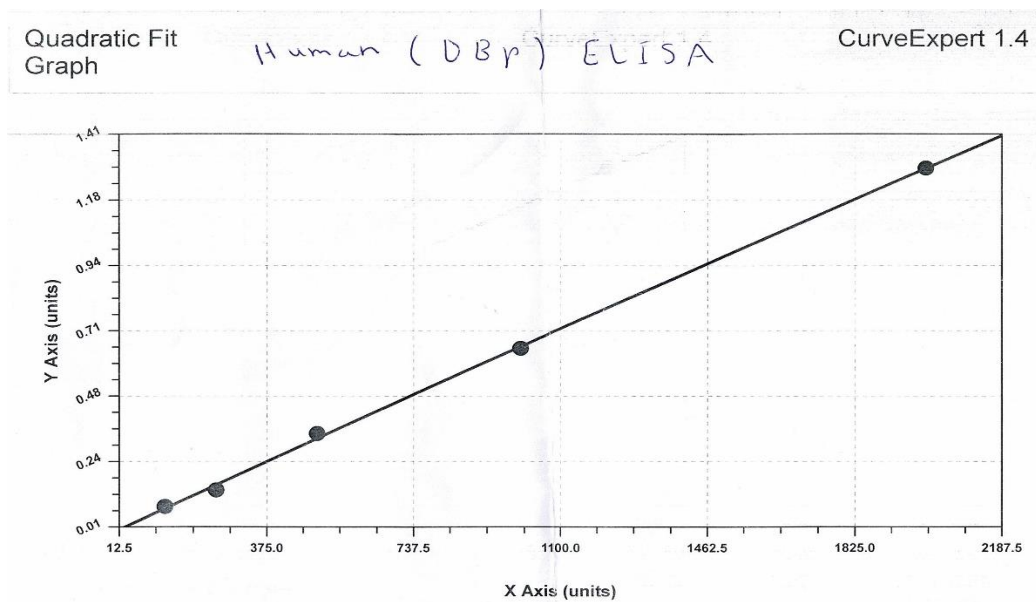

Figure 1: Results of the level of urinary DBP in the samples by ELISA test

Statistical analysis

Statistical analysis was done by SPSS v26 (IBM Inc., Chicago, IL, USA). Quantitative variables were presented as mean and standard deviation (SD) and compared between the two groups utilizing ANOVA (F) test. Qualitative variables were presented as frequency and percentage (%) and were analysed utilizing the Chi-square test. A two tailed P value < 0.05 was considered statistically significant.

Results:

There were no statistically significant differences between the three groups as regard sex distribution (P value=0.150). There were statistically significant differences between the three groups as regard age distribution (P value<0.001). **Table 2**

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***	
							P1	0.215
Range	33-61		33-60		33-50		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

There were no statistically significant differences between the three groups as regard duration of D.M. (p-value=0.880). **Table 3**

Table 3: Duration of Diabetes mellitus (DM) between group I and group II

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

DM: Diabetes mellitus

There were statistically significant differences between the three groups as regard level of creatinine (P value<0.001). There were statistically significant differences between the three groups as regard level of albumin creatinine ratio (P value<0.001)**.** There were statistically significant differences between the three groups as regard level of fasting blood sugar (P value<0.001). There were statistically significant differences between the three groups as regard level of Haemoglobin A1C (P value<0.001). There was statistically significant increase in level of Urinary VDBP in between groups (P value<0.001). **Table 4**

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

		Group I	Group II	Group III	P-value

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

*: 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.

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

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

Correlations		
	Urinary VDBP (µg/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 ≤ 0.05, Cr: Creatinine, ACR: Albumin creatinine ratio, FBS: Fasting blood sugar, HA1C: Hemoglobin A1C, VDBP: Vitamin D Binding Protein.

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 ≥ 125µg/ml/mg corresponding to 77.50% sensitivity and 85.00% specificity. **Figure 2**

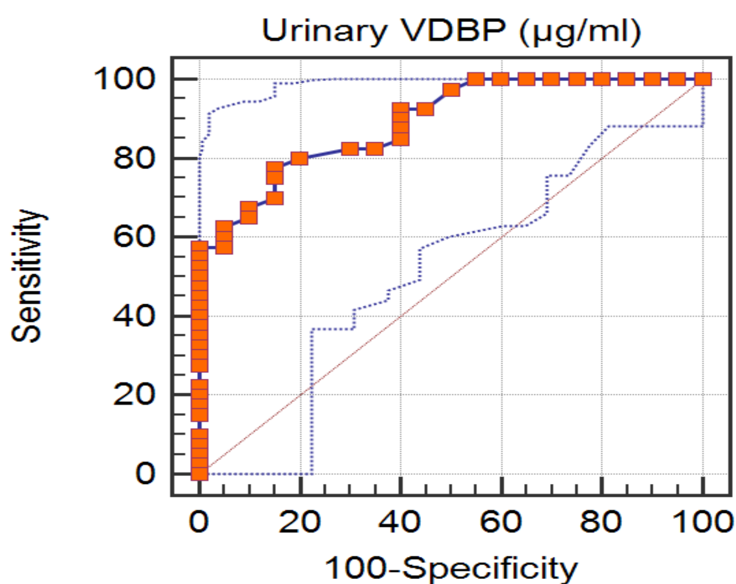

Figure 2: ROC curve of Urinary VDBP between group I and II and group III (healthy group)

Discussion

About 246 million people worldwide had diabetes in 2007, which is projected to increase to 550 million in 2030 [14].

VDBP is a 58-kDa glycoprotein and is present in the serum at a concentration of 300-600 mg/ml [15].

Clinically, a study by Zoidakis and colleagues [16] identified that the reduction in VDBP levels in the urine of patients with invasive bladder cancer was significant, which is consistent with the findings by Li and colleagues [17]. Moreover, Li and colleagues [17] also demonstrated that

the expression levels of UVDBP were positively associated with the pathological classification of bladder cancer. Their results suggested that UVDBP may be a potential non-invasive biomarker for the early diagnosis and effective surveillance of bladder cancer.

Concerning UVDBP our results were in agreement with those of Mirkoviae et al., [18] indicated that the urinary excretion of VDBP may be a novel urinary biomarker of tubulointerstitial damage. 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 studied45 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 AlSel, [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 AlSel [21], negatively with, Age were in agreement with Fawzy and Abu AlSel [21].

Concerning the analysis of our result of an optimum cut-off ,our results rendered an optimum cut-off value of ≥ 125μg/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 rendered an optimum cut-off value of 552.243 ng/mg corresponding to 92.86% sensitivity and 85% specificity, Khodeir et al., [20] the analysis their result of rendered that an optimum cut-off value of UVDBP corresponding to 90.0% sensitivity and 76.7% specificity is appropriate for detecting DN and Fawzy and Abu AlSel, [21] the analysis their result of rendered that 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.

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.

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.

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COMPETING INTERESTS DISCLAIMER:

Authors have declared that no competing interests exist. The products used for this research are commonly and predominantly use products in our area of research and country. There is absolutely no conflict of interest between the authors and producers of the products because we do not intend to use these products as an avenue for any litigation but for the advancement of knowledge. Also, the research was not funded by the producing company rather it was funded by personal efforts of the authors.