



Association of Urinary Vitamin D-binding Protein and Megalin as Biomarkers for Diabetic Nephropathy in Type 2 Diabetes Mellitus in Qatari Patients

Sharif E*, Kitaz N, Hussain A and Alwakeel M

Department of Biomedical Sciences, College of Health Sciences, Qatar University, Doha, Qatar

Abstract

Background: Nephropathy is a common complication of type 2 diabetes mellitus (T2DM). Previous studies revealed that T2DM patients with nephropathy have higher concentrations of urinary Vitamin D Binding Protein (VDBP) that carries vitamin D to the target tissues, and megalin that mediates endocytosis in the proximal tubule than those who are healthy.

Methodology: 196 urine samples with their blood data were obtained from Qatar Biobank, of which 21 samples were measured for VDBP and megalin using enzyme-linked immunosorbent assay (ELISA). They were divided into three groups; group 1 (control group with eGFR \geq 90 mL/min/1.73 m²), group 2 (T2DM patients with eGFR \geq 90 mL/min/1.73 m²) and group 3; (T2DM patients with eGFR < 90 mL/min/1.73 m²).

Results: Urinary VDBP and Megalin levels were non-significantly elevated in T2DM patients with DN (P=0.198) and (P=0.293) respectively. Moreover, a weak negative correlation was observed between the urinary VDBP and Megalin levels with eGFR ($r=-0.326$, P=0.149) and ($r=-0.315$, P=0.165) respectively.

Conclusion: Previous studies revealed that uVDBP and Megalin are potential biomarkers for DN in T2DM patients. However, the current study reveals that urinary VDBP and megalin levels were non-significantly elevated in T2DM patients with DN. Furthermore, eGFR showed a weak negative correlation with urinary VDBP and megalin levels. However, it is suggested that these results could be due to some limitations. Further tests should be performed on larger sample size to confirm the association of Megalin and VDBP in T2DM nephropathy.

Keywords: Type 2 diabetes mellitus; Nephropathy; Estimated glomerular filtration; Megalin; Vitamin D binding-protein

Introduction

Diabetes mellitus

Diabetes mellitus (DM) is a chronic disorder that is characterized by multiple metabolic dysfunctions. This includes abnormal insulin secretion or insulin action or both of them, which can lead to hyperglycemia [1], and abnormal metabolism of carbohydrates, proteins and lipids [2]. DM has two types, Type 1 Diabetes Mellitus (T1DM), which is an insulin-dependent form of DM and Type 2 Diabetes Mellitus (T2DM), which is a non-insulin dependent form of DM. T2DM is more common, in which it occupies 90%-95% of the diabetic patients [3]. It affects people from both developing and developed countries that have an increased rate of diabetic-related mortality and morbidity. T2DM is a serious chronic disease that develops due to different hereditary and environmental factors, where most of its patients are obese or have a high percentage of body fat distributed among the abdominal region. Moreover, other modifiable risk factors could be included, such as the age and the absence of physical exercise [3]. The prevalence of the disease is still increasing; thus, it is important to introduce efficient strategies in order to prevent having any new cases and to enhance the early detection of the disease. Furthermore, this will prevent different complications of the disease, which includes kidney and heart diseases and retinopathy [3].

Epidemiology

DM is a leading cause of mortality and morbidity [1]. It is a common disease in Qatar. According to Hamad Medical Corporation (HMC) (2017), the prevalence of diabetes is 17% and 11-23% is at risk of having diabetes [2]. Moreover, Qatar is considered to be one of the countries that

have an increased rate of glucose intolerance in 17% of its population. Furthermore, in 2012, it was reported that the prevalence of Qatari T2DM patients was 16.7%, and by 2050, it is predicted to reach 24%, where most of T2DM are aged from 18 to 64 years old. Additionally, the prevalence of physical inactivity, obesity and active smoking were 45.9%, 41.4% and 16.4% respectively [4]. Obesity is considered to be a key factor that affects two-thirds of the incidence of T2DM Qatari patients. Therefore, evaluating the future of T2DM in Qatar is crucial to be notified to control the prevalence of the disease by addressing new preventive methods, early detection of the disease and therapeutic interventions [4]. Moreover, it is a worldwide metabolic disease in which its prevalence is rising to more than one million new cases annually in the USA [1]. Additionally, in 2015, diabetes results in up to 5 million deaths worldwide [5-13]. T2DM incidence differs significantly from one geographical area to another according to the variation of environmental, lifestyle and genetic risk factors. The prevalence of T2DM in adult patients is expected to rise in the next decades and to increase greatly in developing countries [14].

*Corresponding author: Sharif E, Department of Biomedical Sciences, College of Health Sciences, Qatar University, Doha, Qatar, Tel: +97444034788; E-mail: e.sharif@qu.edu.qa

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Normal physiology of pancreas

Regulating the glucose level in the blood involves different organs, where the main organ is the pancreas, which controls this process by secreting either insulin or glucagon hormones that work against each other. Insulin is produced by the β -cells of the islets of Langerhans in the pancreas. It is secreted as a result of increased blood glucose level due to food intake in order to reduce the glucose level to the normal level, thus, it is called a hypoglycemic agent, noticing that it is the only hormone that performs this process. Insulin allows the glucose to enter the liver, muscle and adipose cells. Moreover, it controls the glucose level by increasing different processes that include glycolysis, glycogenesis (storing glucose in the liver by converting it into glycogen) and lipogenesis (converting glucose into fatty acids), and prohibiting glycogenolysis. Glucagon is produced by the β -cells of the islets of Langerhans in the pancreas. It is secreted as a result of decreased blood glucose level due to fasting and stress conditions in order to raise the glucose level to the normal level, thus, it is called a hyperglycemic agent. Glucagon stimulates glycogenolysis, which converts the glycogen that is stored in the liver into hepatic glucose and increases gluconeogenesis (synthesis of glucose from other sources rather than carbohydrates) [11].

Causes of T2DM

T2DM in the non-insulin dependent form of DM that is influenced by genetic factors. However, obesity, age, and lifestyle are independent risk factors that could accelerate the progression of T2DM in those who are genetically susceptible [11-13]. T2DM occurs as a result of insulin resistance and/or impaired pancreatic β -cell function that results in an insufficient insulin production. Consequently, the transportation of glucose into the liver, fat and muscle cells will reduce, which results in hyperglycemia [14]. Furthermore, it has been recognized that cell dysfunction is also involved in the etiology of T2DM. It results in the production of excessive levels of glucagon and hepatic glucose that also leads to fasting hyperglycemia [14].

Diagnosis of diabetes mellitus

DM could be diagnosed by testing random plasma glucose level, fasting plasma glucose level FPG; which is preferred over random plasma glucose level, Oral Glucose Tolerance Test OGTT; which determines the ability of the cells to absorb glucose by measuring glucose 2 hours after taking 75 g of glucose, and Hemoglobin A1c, HbA1c, which can be monitored over the period of two to three months of the diabetic patients (Table 1). Symptoms and signs, and the onset of the disease should be considered in order to differentiate between T1DM and T2DM, where T2DM is characterized by adult onset, polydipsia, polyuria, weight loss, hypertension, increased body mass index BMI: overweight 25 kg/m², dyslipidemia, and signs of insulin resistance that can be confirmed by further tests [11], such as testing C-peptide, which is produced with each insulin molecule and has longer half-life time than insulin, thus, it is used to evaluate the function of pancreatic β -cells and to determine the insulin level in the blood [15].

	FPG	OGTT	HbA1c
Normal	70-99 mg/dL	<140 mg/dL	<5.7%
Pre-diabetes	100-125 mg/dL	140-199 mg/dL	5.7%-6.4%
Diabetes (Need to be confirmed)	≥ 126 mg/dL	≥ 200 mg/dL	≥ 6.5%

Table 1: Shows different diagnostic tests of diabetes.

Complications of diabetes mellitus

The hyperglycemia that result of T2DM can lead to macrovascular

and microvascular complications. Macrovascular complications include hyperlipidemia, hypertension, coronary artery disease, heart attacks, strokes, and peripheral and cerebral vascular system diseases [16]. While microvascular complications include nephropathy, retinopathy and neuropathy. One of the most common complications of DM is Diabetic Nephropathy (DN), which will be discussed in detail [3].

Diabetic nephropathy

DN, which is also called Diabetic Kidney Disease (DKD) [17], is found in approximately 30-40% of diabetic patients. It is described by low Glomerular Filtration Rate (GFR) resulting in proteinuria. Also, it is a long-term complication that depends on the duration of diabetes being a chronic disease [5]. Unfortunately, controlling DM will not totally prevent the Chronic Kidney Disease (CKD) that leads to kidney failure at the advanced stage. Nephropathy is considered to be the most essential cause of chronic renal failure worldwide [18]. A study that was conducted by HMC between May 2001-2002 that included 225 Qatari diabetic patients showed that 40.9% of them have nephropathy [19]. Later between May 2011 to January 2013, another study included 1633 Qatari diabetic patients aged above 20 years, revealed that the prevalence of diabetic nephropathy was 12.4% [20]. Recently, in 2017, HMC revealed that 45.3% of 2000 diabetic patients were suffering from nephropathy [2]. Moving to the Gulf region, in Saudi Arabia, a study was conducted in 2004 about T2DM patients who were attending a primary health care centre in Abha city revealed that about 54.3% had proteinuria. Later in 2013, a cross-sectional study that includes 54,670 Saudi T2DM patients who were members of Saudi National Diabetes Registry indicated that the prevalence of DN was 10.8%. In Kuwait, between June 2006 and May 2007, a study included El-Fahaheel primary health-care centre T2DM patients showed that the prevalence of macro-albuminuria was 58.2%. Another study in Oman between 2010 to 2011 involved T2DM Omani patients revealed that 42.5% of them had proteinuria as a result of DN [21]. Moreover, about 24 million individuals in the United States have DM and approximately 180,000 of them are suffering from kidney failure due to diabetes and DKD. [18]. DN is a diabetic microvascular complication, which results from hyperglycemia that leads to oxidative stress, which accelerates the formation of Advanced Glycation End products (AGEs) in the circulation [22]. AGEs are the end products of Non-Enzymatic Glycation (NEG), in which sugars are bound to proteins. This will result in multiple cellular damages [23]. At the early stage of DN, AGEs cause glomerular endothelial dysfunction and alter the Extra Cellular Matrix (ECM) composition that leads to mesangium expansion and thickening of the Glomerular Basement Membrane (GBM) [22,23]. Furthermore, AGEs enhance angiotensin II activity [23], which causes vasoconstriction of efferent arteriole and vasodilation of afferent arteriole. This results in a high intraglomerular pressure that leads to hyperfiltration [24]. AGEs accumulation in the extracellular matrix components of kidney capillaries leads to podocytes injury [23], where podocytes have a role in maintaining glomerular permeability rate [22], in which they are considered to be as a barrier that prevents the plasma proteins to pass into the filtrate. Damaging podocytes includes their detachment and depletion, which results in increased vascular permeability of albumin, in which in the normal state, the glomerular filtrate is almost free from albumin [23]. This will lead to renal filtration dysfunction [22] that is characterized by low glomerular filtration rate. This usually indicates progressive DKD that is associated with the development of proteinuria that is described by micro-albuminuria and macro-albuminuria in diabetic patients [23]. Furthermore, this will overload the endocytosis of filtered AGEs in the epithelial cells of the proximal tubule that leads to cells toxicity and tubulo interstitial injury [9]. This is shown in Figure 1. DN is usually

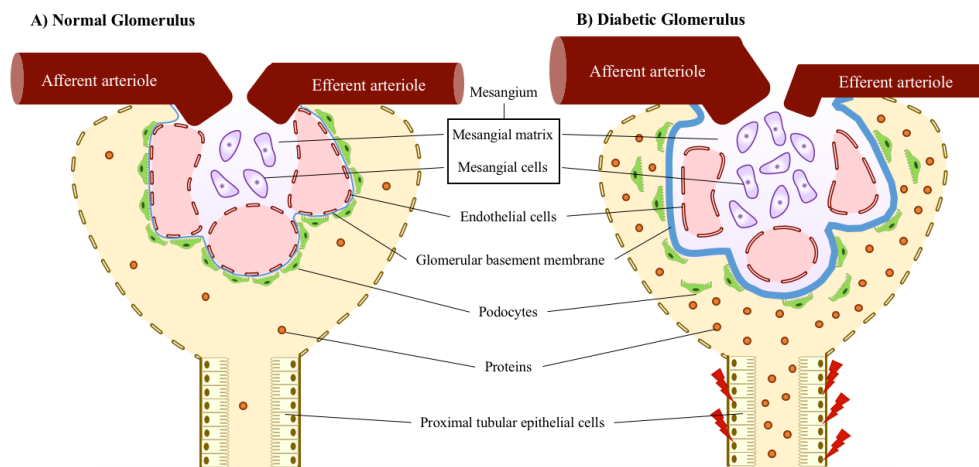


Figure 1: Shows the differences between normal glomerulus and diabetic glomerulus (A) Shows the normal structure of the glomerulus in healthy individuals. (B) Shows different structural abnormalities in diabetic patients as a result of diabetic nephropathy. It includes glomerular endothelial dysfunction, mesangium expansion, thickening of the glomerular basement membrane, vasoconstriction of efferent arteriole, vasodilation of afferent arteriole and podocytes detachment. In addition to proximal tubule epithelial cells injury. Where all of these structural abnormalities lead to low glomerular filtration rate and proteinuria.

diagnosed according to the presence of microalbuminuria in urine. This was useful in the early detection of the microvascular complications of the disease. Nevertheless, it is unable to predict DKD adequately, especially in young diabetic patients or those who are non-albuminuric. Further measurements were proposed to discover glomerular or/and tubular dysfunction [17]. Estimated Glomerular Filtration Rate (eGFR) is an equation that is used to calculate the GFR instead of direct testing of the urine sample. It is mainly based on serum creatinine, gender, age, and ethnicity, where it is used to reveal the glomerular function and the kidney status. Probably, blood testing of kidney function tests and urinalysis will be performed initially, where the abnormal results may give a hint about the general kidney status [11].

Treatment of T2DM

T2DM is mainly controlled by having a healthy lifestyle that is based on having diet and exercising to reduce and control obesity, glucose level, dyslipidemia, hypertension, and other complications [11-14]. Some drugs could be used including metformin, sulfonylureas, thiazolidinediones, alpha-Glucosidase inhibitors, incretin-based therapies and insulin therapy. Metformin is an insulin sensitizer, which is the first oral therapeutic option in T2DM patients. It increases the insulin sensitivity of the cells and decreases the production of hepatic glucose, the absorption of glucose in the intestine, and the level of triglycerides and low-density lipoprotein (LDL) [25]. Sulfonylureas stimulate the production of endogenous insulin. However, it can cause hypoglycemia, especially in elderly patients. Thiazolidinedione is also an insulin sensitizer, which includes pioglitazone that well-tolerated in older patients and could be used by patients with renal impairment but not by patients with heart failure. Alpha-Glucosidase Inhibitors includes Acarbose, Miglitol and Voglibose are not commonly used to treat T2DM patients and should not be given to in cases of significant renal impairment. However, they are safe and effective, especially for postprandial hyperglycemia [14]. Incretins are intestinal secreted hormones that stimulate insulin production and inhibit postprandial glucagon production. Incretin-based therapies that include Glucagon-Like Peptide 1 (GLP-1), is an ideal therapy of T2DM due to its good tolerability, efficacy, weight loss and low risk of causing hypoglycemia. It has a positive influence on central nervous, cardiovascular, inflammation, hepatic health and sleep system. Insulin therapy is an

effective therapy for glycemic control. Insulin can inhibit the secretion of the hepatic glucose and increase the utilization of postprandial glucose. Moreover, it can improve the insulin sensitivity of the cells and the secretory function of the β -cell by reducing the glucose level in the blood, hence, preventing the glucose toxicity [3].

Vitamin D binding protein

Vitamin D binding protein (VDBP) is also known as a group-specific component, which belongs to the Gc globulin family that albumin belongs to as well. VDBP is a 58 kDa glycosylated alpha-globulin, which is made up of 458 amino acids. It has different binding regions that enhance its functions, such as binding regions of vitamin D, fatty acid, and actin and cell surface [6]. VDBP is mainly secreted by the liver in quite constant rates during life. However, it has been observed that the concentration of VDBP increased under some condition like pregnancy, where the estrogen level is elevated and at day-time [6]. Other tissues/organs that can produce VDBP in low concentrations include abdominal fat, testis, and kidney. Also, it is expressed on the surface of immunocytes [26]. The main function of VDBP is to carry and transport the circulating vitamin D and its metabolites to the target tissues. In addition, the bioavailability of 1,25-dihydroxyvitamin D (1,25 (OH) 2D), the active form of vitamin D and its precursor 25-hydroxyvitamin D (25 (OH) D) is supported by VDBP. Furthermore, it has an important role in the biosynthesis of 1,25 (OH) 2D in renal proximal tubules [7]. Indeed, VDBP offers less than 5% of its binding sites to be bound with vitamin D metabolites. Since VDBP has a stronger affinity for vitamin D metabolites than albumin, 85% to 95% of them are bound to VDBP. The rest of vitamin D metabolites (bioavailable) can bind to albumin or circulate in the blood (<5%) [6]. Moreover, the rest of VDBP are involved with other functions that are essential for the biological processes, which includes fatty acid transportation, actin scavenging, chemotaxis, osteoclasts stimulation and macrophages activation. It is also associated with T and B immune cells surfaces [1]. In addition, VDBP has an important role in immune response and inflammatory processes. Furthermore, bioinformatics analysis determined that Gc content play a role in apoptosis and epidermal growth factor receptor [26]. In the recent years, urinary VDBP (uVDBP) is considered to be as a novel biomarker of DN, where it enhances its detection in T2DM patients as discussed in which shows the previous studies that measure the uVDBP in T2DM.

Megalin

Renal Proximal Tubule Epithelial Cells (PTEC) includes receptors that mediate endocytosis, which have a vital role in reabsorbing and metabolizing different substances including proteins and carrier-bound vitamins from glomerular filtrate. Any damage in the endocytosis process will lead to the passing of those substances into the urine, which results in proteinuria. This significantly indicates kidney and cardiovascular diseases [27,28]. Megalin is a 600 kDa glycoprotein with 4,655 amino acids that belongs to the LDL receptor family, in which it is encoded by LDL Related Protein 2 (LRP2) gene [10]. Megalin is mainly expressed in PTECs, specifically in its apical membranes, which is in contact with the lumen rather than the bloodstream. It is considered to be the most vital receptor in PTECs that is classified as a scavenger receptor, which mediates endocytosis of different ligands [29]. These ligands involve different binding proteins including Vitamin-Binding Proteins [29] (e.g. VDBP) [28], apolipoproteins, enzyme and enzyme inhibitors, hormones and hormone precursors, stress-and immune-response-related proteins, drugs and toxins [29]. Furthermore, megalin is present in small portions in lysosomes when assigned for degradation [10] and in different epithelial cells, such as glomerular podocytes, thyroid and parathyroid cells, type II pneumocytes and choroid plexus [29]. The renal proximal tubule shows a wide apical endocytic apparatus, which contains a network of molecules that collaborate with megalin to reabsorb the ligands from the glomerular filtrate, which explains the fact of human urine samples that are almost free from proteins under normal conditions. However, proteins are not the only vital substance that undergoes reabsorption, a variety of nutrients and molecules including vitamins with their binding proteins (e.g. VDBP) are also reabsorbed to be returned to the bloodstream [29]. Moreover, megalin has a role in taking up pathological and toxic molecules to PTECs or overloading endocytosis, which results in cellular damages that lead

to kidney diseases [28-30]. In 2012, urinary megalin was considered to be as a novel biomarker of DN, in which it is a scavenger receptor that mediates the endocytosis of the AGEs and their degradation in PTECs [9-23] that leads to will lead to tubular injury [8] and shedding of megalin receptors through urine in diabetic patients [10]. Megalin enhances the detection of DN in T2DM patients as discussed in which shows the previous study that measures the urinary megalin in T2DM.

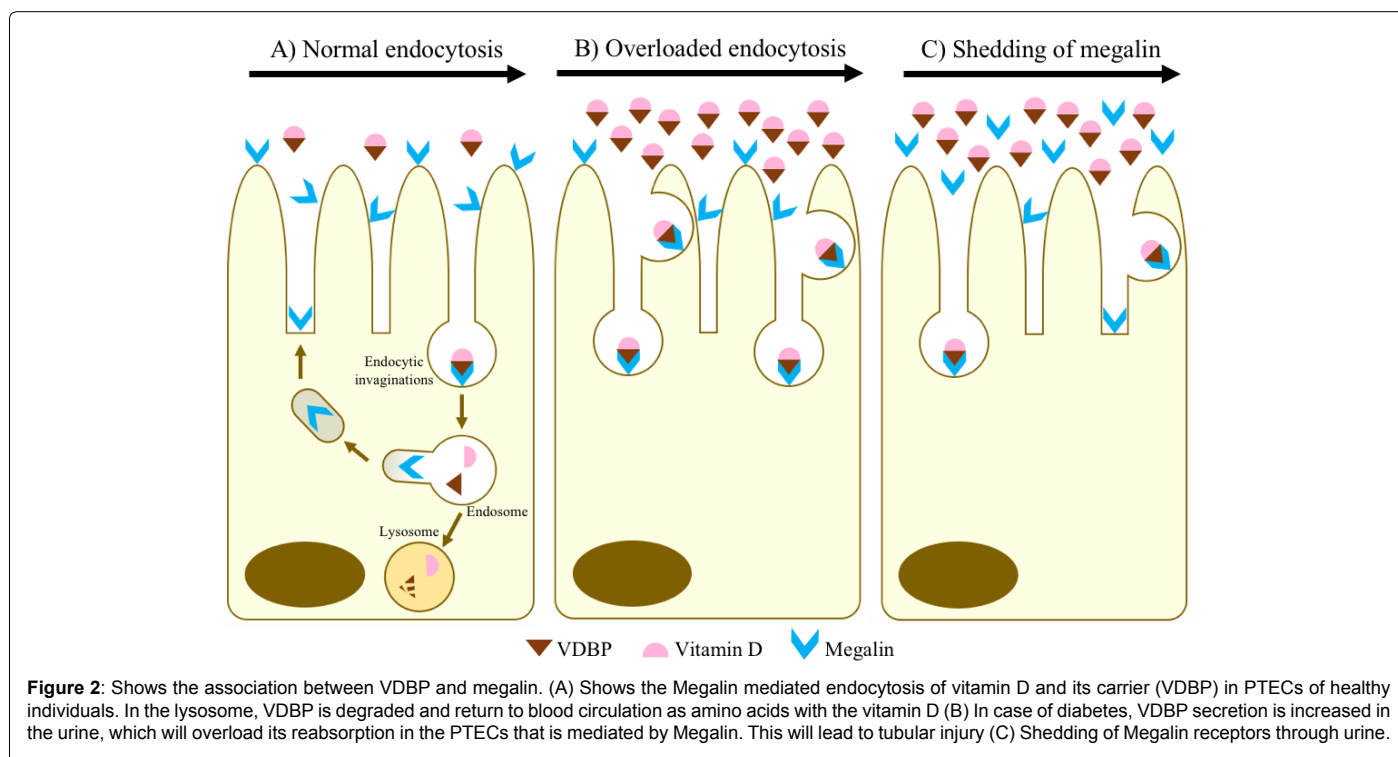
Association between VDBP and Megalin

The main function of VDBP is to carry the circulating vitamin D and its metabolites to the target tissues [7]. As a result of DN, VDBP secretion is increased in the urine. This will overload its reabsorption in the renal proximal tubules that is mediated by different receptors including megalin [8], which is a scavenger receptor that mediates the endocytosis in the PTECs of different ligands [9]. Endocytosis is an energy-consuming process, where in case of diabetes. It is overloaded by the increased secretion of uVDBP, which will lead to tubular injury [8] and shedding of megalin receptors through urine [10]. This association is shown in Figure 2.

Materials and Methods

One hundred ninety-six urine samples with their blood data were obtained from Qatar Biobank and stored at -80°C till further processing. Twenty-one samples were measured for VDBP and megalin using enzyme-linked immunosorbent assay (ELISA) (DVDBPOB for Human VDBP and LS-F11978 for Human LRP2/Megalin). This project was approved by Institutional Review Board (IRB) of Qatar Biobank (Approval number: Ex-2017-QBB-RES-ACC-0082-0029).

According to HMC, Modification of Diet in Renal Disease (MDRD) equation [31] was used to calculate the estimated glomerular filtration rate (eGFR) of all samples



$$eGFR(\text{mL}/\text{min}/1.73\text{ m}^2) = 175 \times \left(\frac{\text{Serum Creatinine}}{88.4} \right)^{-1.154} \times (\text{Age})^{-0.203} \times \left(\begin{array}{l} 0.742 \\ \text{if female} \end{array} \right) \times \left(\begin{array}{l} 1.212 \\ \text{if black} \end{array} \right)$$

The samples were divided into three groups according to the level of the eGFR [11]; group 1 (control group with eGFR ≥ 90 mL/min/1.73 m²), group 2 (T2DM patients with eGFR ≥ 90 mL/min/1.73 m²) and group 3; (T2DM patients with eGFR < 90 mL/min/1.73 m²).

Study subjects

T2DM patients were included in the study. Pregnant ladies, smokers, and patients who are taking vitamin D supplements or any drugs (except diabetic medication), and having inflammations, tumors, or infections were excluded from the study. For the control group, individuals with normal lab results of HbA1c, C peptide protein, insulin, creatinine, urea, bilirubin, total protein, and albumin were used. Whereas patients who have diabetes, kidney and liver diseases, pregnancy, smoking, inflammations, urinary system disorders, tumors, infections and are taking any supplements or medications were excluded from the study. After obtaining the samples from Qatar Biobank, another exclusion criteria was performed. This is shown in Figure 3.

Determination of VDBP

The concentration of VDBP in urine samples was measured using human vitamin D BP Quantikine ELISA kit (catalog #: DVDBP0B; Lot #: P140502; R&D Systems, Minneapolis, MN, USA). First, the microplate was pre-coated with monoclonal antibodies specific for human VDBP. Standards and samples were pipetted in each well. In case VDBP is present, then it will bind to the immobilized antibody. Furthermore, washing step was performed to remove any unbound substances. After that, an enzyme-linked monoclonal antibody that is specific for VDBP (conjugate) was added. Later, washing was performed again to remove any unbound conjugate. Furthermore, the substrate was added, which contain color reagent A (stabilized hydrogen peroxide) and color reagent B (tetramethylbenzidine; stabilized chromogen) and the color was developed in proportion to the amount of VDBP bound in the initial step. Finally, stop solution was added to stop the reaction and the intensity of the color was measured using microplate reader at a wavelength 450 nm.

Determination of megalin

The concentration of megalin in urine samples was measured using human LRP2/megalín sandwich ELISA kit (catalog #: LS-F11978; Lot #: 115033; LifeSpan Biosciences, Seattle, WA, USA). First, the wells of the microtiter plate were pre-coated with a capture antibody that is specific to megalin. In each well, standards and samples were pipetted.

If the megalin is present, then it will form a binding with the capture antibody. Furthermore, washing step was performed to remove any unbound substances. After that, detection reagent A was added, which is a biotin-conjugated detection antibody that binds to the captured antigen. Later, washing was performed again to remove any unbound conjugated antibody. Furthermore, detection reagent B was added, which is an Avidin-Horseradish Peroxidase (HRP) conjugate that binds to the conjugated antibody. Another washing step was performed again to remove any unbound Avidin-HRP conjugate. Additionally, the 3,3',5,5'-Tetramethylbenzidine (TMB) substrate was added. A color development was observed due to the reaction that had occurred between the TMB substrate and HRP enzyme, which was terminated by adding the stop solution (sulfuric acid). Finally, the color intensity of the microtiter plate was measured by using microplate reader at a wavelength of 450 nm.

Statistical analysis

Statistical analyses were performed using SPSS 24 software. Data presented the median and interquartile range (25-75%) for the non-normally distributed continuous data. However, the mean and standard deviation were calculated for normally distributed continuous data. The comparison between groups for nonparametric values was analyzed by the Kruskal Wallis followed by post hoc test. While for parametric values, ANOVA was used. Moreover, correlation analysis (Bivariate) was used to determine the correlation between eGFR, megalin, uVDBP, vitamin D and the duration of T2DM using Spearman correlation coefficient. All tests were two-tailed and a P-value of ≤ 0.05 was considered statistically significant.

Results

Clinical characteristics

Table 2 demonstrates the study subjects that were divided into three groups according to eGFR. G1; which is a healthy control group with normal eGFR ≥ 90 ml/min/1.73 m², G2; which presents T2DM patients with normal eGFR ≥ 90 ml/min/1.73 m² and G3; which is T2DM patients with abnormal eGFR < 90 ml/min/1.73 m².

Correlations between different variables

The relationship between the main variables in this study was determined, which includes eGFR, megalin, uVDBP and the duration of T2DM. Megalin and uVDBP have non-significant negative linear relationships with eGFR ($r = -0.315$, $P = 0.165$) and ($r = -0.326$, $P = 0.149$) respectively. While the uVDBP has a non-significant positive linear relationship with duration of T2DM ($r = 0.054$, $P = 0.861$) and megalin

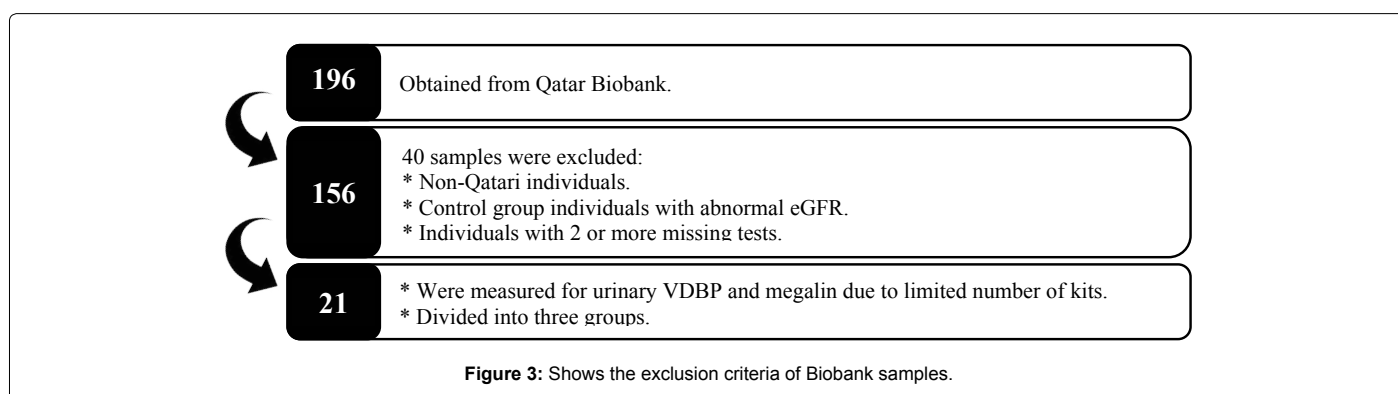


Figure 3: Shows the exclusion criteria of Biobank samples.

Variables	G1	G2	G3	P-value (G1 & G2)	P-value (G1 & G3)	P-value (G2 & G3)
n=21	7	7	7			
Female	4	3	4	-	-	-
Male	3	4	3	-	-	-
Age	30 (26-46)	59 (50-63)	45 (37-59)	-	-	-
eGFR (ml/min/1.73m ²)	97.1 (94.9-105.4)	100.8 (95-105.6)	86.4 (77.6-88.9)	1	0.005*	0.004*
VDBP (ng/ml)	20.3 (16.-22.1)	18.9 (14.2-48.4)	52.4 (32.8-61.4)	0.198	0.198	0.198
Megalin (ng/ml)	12.9 (0.84-70.4)	1.5 (0-7.3)	18.9 (3.5-305.8)	0.293	0.293	0.293
Serum creatinine (umol/L)	(67.3 ± 8.15)	(64 ± 10.16)	(73.8 ± 9.4)	0.372	0.372	0.372
Urea nitrogen (mmol/L)	3.1 (2.6-3.5)	4.9 (4.4-6.1)	4.7 (3.7-5.2)	0.006*	0.06	1
Uric acid (umol/L)	259 (214.7-362.5)	286 (244.2-339.7)	279 (243.2-411.5)	0.953	0.953	0.953
Total protein (gm/L)	73 (71.2-76)	71 (66.7-72.7)	72 (71-72)	0.237	0.237	0.237
Albumin (gm/L)	47 (44-47)	44 (41.5-46.7)	44 (43.2-45)	0.34	0.34	0.34
Cholesterol (mmol/L)	4.8 (4.1-5)	4.6 (3.7-5.2)	5.4 (4.4-6.4)	0.293	0.293	0.293
Triglyceride (mmol/L)	1.2 (0.6-1.3)	1.1 (0.95-1.4)	1.9 (0.93-2.6)	0.609	0.609	0.609
LDL (mmol/L)	2.5 (2.1-3)	2.8 (1.9-3.1)	3.9 (2.8-4.1)	0.13	0.13	0.13
HDL (mmol/L)	1.2 (1.1-1.7)	1.3 (1-1.4)	0.9 (0.9-1.2)	0.188	0.188	0.188
Homocysteine (umol/L)	8.5 (7.5-9.8)	9.1 (7.9-10.4)	7.9 (7.5-8.5)	0.743	0.743	0.743
Insulin (mcunit/mL)	8 (6.5-18.7)	11.8 (7.3-12.5)	9 (4.6-12.9)	0.833	0.833	0.833
C-peptide of insulin (ng/mL)	2.2 (1.5-3.1)	2.2 (1.7-2.6)	1.4 (1.3-2.1)	0.256	0.256	0.256
HbA1c %	5.1 (4.9-5.6)	7 (6.3-7.5)	7.8 (6.1-11.8)	0.016*	0.005*	1
Glucose (mmol/L)	4.8 (4.5-5.3)	7 (6.1-10)	7.2 (6.3-11.6)	0.011*	0.006*	1
BMI (Kg/m ²)	25.7 (22.8-31.1)	28.1 (27.4-31.5)	31.9 (27.1-35.6)	0.237	0.237	0.237
Duration of DM (Years)	-	4 (3-15)	3 (2.2-3.7)	-	-	0.237

Serum creatinine is the only parametric variable that is expressed as mean ± SD. While median and (interquartile range) are expressed for all the remaining non-parametric variables.
 eGFR, uVDBP, megalin, kidney profile tests (serum creatinine, urea nitrogen, uric acid), liver profile testes (total protein and albumin), lipid profile tests (cholesterol, triglyceride LDL, high-density lipoprotein [HDL]), homocysteine, insulin, c-peptide of insulin, HbA1c, glucose, BMI, and the duration of T2DM were compared among the three studied groups.
 * P-value ≤ 0.05 is considered as clinically significant.

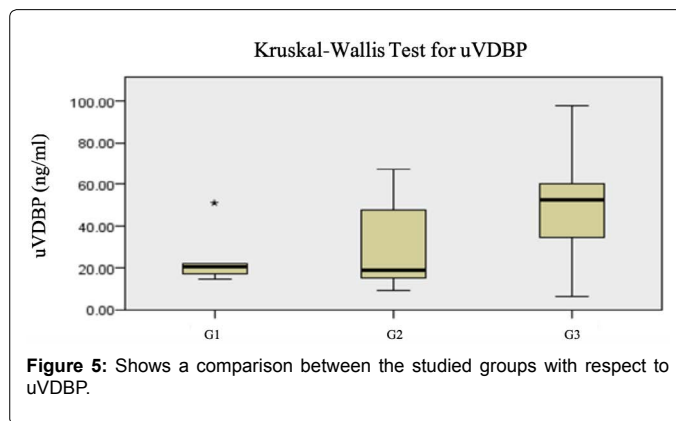
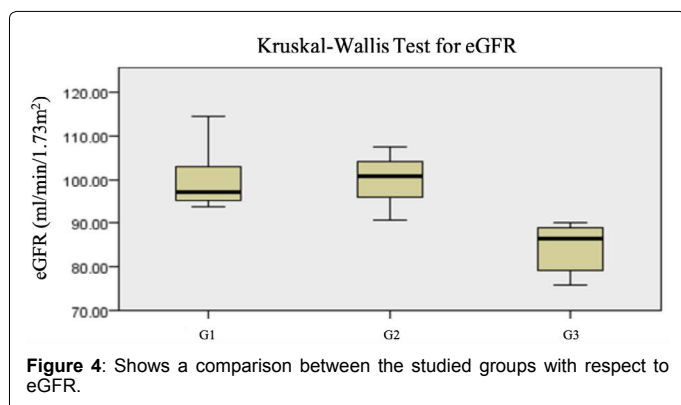
Table 2: Shows the clinical characteristics of different variables for G1, G2 and G3 and the p-value between the groups.

($r=0.001$, $P=0.998$). Furthermore, the duration of T2DM has non-significant positive linear relationships between megalin ($r=0.143$, $P=0.642$) and eGFR ($r=0.295$, $P=0.328$) (Table 3).

Name of correlation	Correlation Coefficient (r)	P-value
eGFR and uVDBP	-0.326	0.149
eGFR and megalin	-0.315	0.165
Duration and megalin	0.143	0.642
Duration and uVDBP	0.054	0.861
uVDBP and megalin	0.001	0.998

Determined by Spearman correlation coefficient using Bivariate correlation analysis. Two-tailed P-value ≤ 0.05 is clinically significant.

Table 3: Shows the correlation coefficient and significance between eGFR, megalin, uVDBP and the duration of T2DM.



Comparison between the studied groups with respect to eGFR

Figure 4 shows the eGFR in G3 (86.4 (77.6-88.9)), which is significantly lower than G1 (97.1 (94.9-105.4), $P=0.005$) and G2 (100.8 (95-105.6), $P=0.004$). However, there was no significant difference between G1 and G2 ($P=1.00$).

Comparison between the studied groups with respect to uVDBP

Figure 5 reveals the uVDBP concentrations in G3 (52.4 (32.8-61.4)), which is non-significantly higher than G1 (20.3 (16-22.1), $P=0.198$) and G2 (18.9 (14.2-48.4), $P=0.198$). Additionally, there was no significant difference when comparing G1 with G2 ($P=0.198$).

Comparison between the studied groups with respect to megalin

Figure 6 demonstrates that megalin concentrations in G3 (18.9 (3.5-305.8)) is non-significantly higher than G1 (12.9 (0.84-70.4), $P=0.293$) and G2 (1.5 (0-7.3), $P=0.293$). Moreover, there was no significant difference between G1 and G2 ($P=0.293$).

Comparison between the studied groups with respect to HbA1c

Figure 7 shows that Hb1Ac concentrations is increased in G3 (7.8 (6.1-11.8)) significantly with G1 (5.1 (4.9-5.6), $P=0.005$) and non-significantly with G2 (7 (6.3-7.5), $P=1.00$). In addition, G2 Hb1Ac concentrations is significantly higher than G1 ($P=0.016$).

Comparison between the studied groups with respect to glucose level

Figure 8 reveals that glucose level in G1 (4.8 (4.5-5.3)) is significantly lower than G3 (7.2 (6.3-11.6), $P=0.006$) and G2 (7.2 (6.3-11.6), $P=0.011$). However, G3 and G2 show a non-significant high glucose level ($P=1.00$).

Comparison between the studied groups with respect to the duration of T2DM

Figure 9 demonstrates the duration of T2DM in G3 (3 (2.2-3.7)) and G2 (4 (3-15)), in which G3 is non-significantly lower than G2 ($P=0.237$).

Comparison between the studied groups with kidney profile tests

The kidney profile tests of the measured serum creatinine, urea nitrogen and uric acid showed non-significant differences among the three groups. However, as shown in Figure 10 urea nitrogen concentration in G2 (4.9 (4.4-6.1)) is elevated significantly compared to G1 (3.1 (2.6-3.5), $P=0.006$) and non-significantly compared with G3 (4.7 (3.7-5.2), $P=1.00$). However, the difference between G1 and G3 was non-significant ($P=0.060$).

Comparison between the studied groups with other variables

Lipids profile tests (cholesterol, triglyceride, LDL and HDL), liver profile tests (total protein and albumin), homocysteine, insulin, C-peptide of insulin and BMI were measured for all the three groups in which all of them shows non-significant differences among each other's.

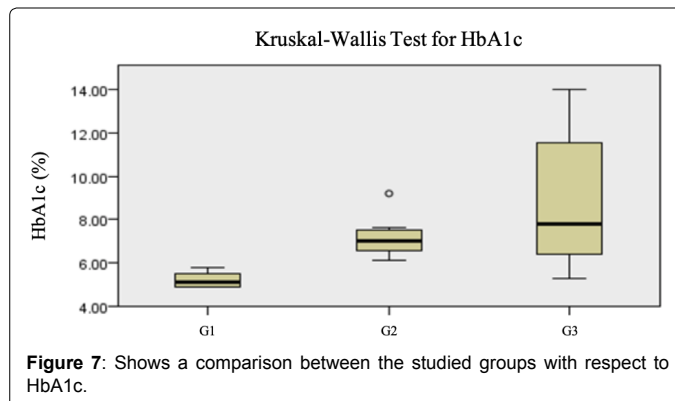


Figure 7: Shows a comparison between the studied groups with respect to HbA1c.

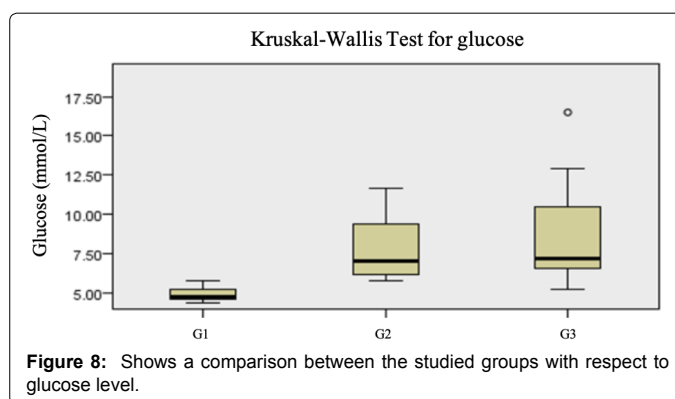


Figure 8: Shows a comparison between the studied groups with respect to glucose level.

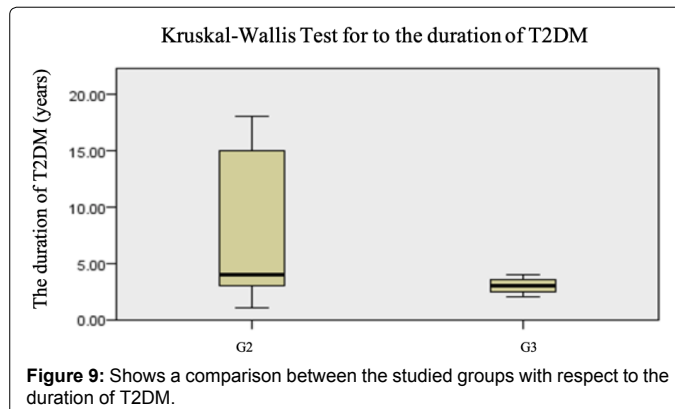


Figure 9: Shows a comparison between the studied groups with respect to the duration of T2DM.

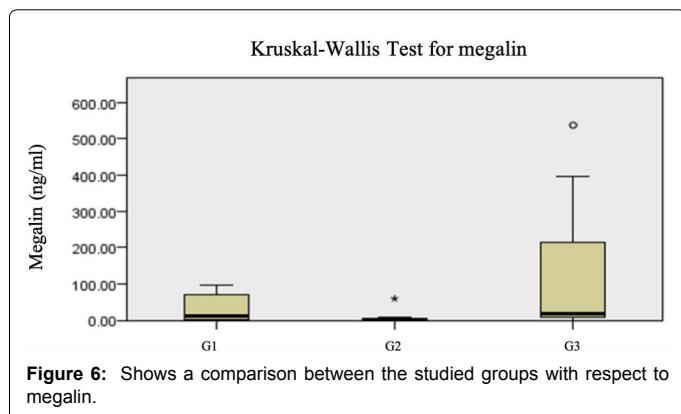


Figure 6: Shows a comparison between the studied groups with respect to megalin.

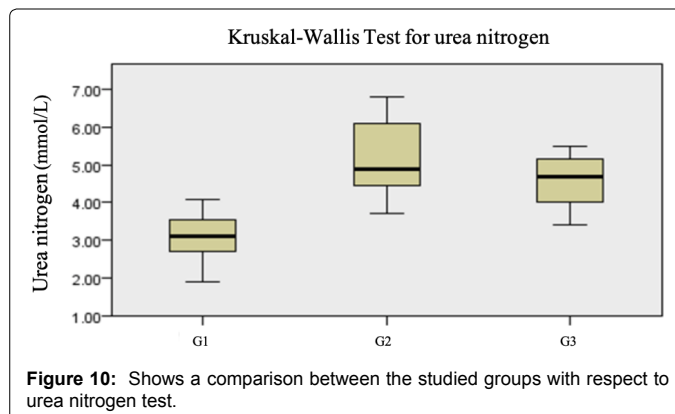


Figure 10: Shows a comparison between the studied groups with respect to urea nitrogen test.

Discussion

DM is a leading cause of mortality and morbidity that is a common disease in Qatar [1, 2]. T2DM is more common than T1DM, in which it occupies 90%-95% of the diabetic patients [3]. One of the most common complications of DM is DN [3]. DN is found in approximately 30-40% of diabetic patients. It is described by low glomerular filtration rate (GFR) resulting in proteinuria. Also, it is a long-term complication that depends on the duration of diabetes [5]. DN is usually diagnosed according to the presence of albuminuria in urine. Moreover, eGFR is used to reveal the glomerular function and the kidney status. According to previous studies that were conducted in five different regions for uVDBP, it was hypothesized that patients with DN have

higher concentrations of urinary VDBP and megalin than those who are healthy. Therefore, this research is aimed to study the uVDBP and megalin concentrations as biomarkers for DN in Qatari T2DM patients, where the study subjects (n=21) including males and females were divided into three groups based on their eGFR that indicates the progression of DN. G1; which is a healthy control group with normal eGFR ≥ 90 ml/min/1.73 m², G2; which presents T2DM patients with normal eGFR ≥ 90 ml/min/1.73 m² and G3; which is T2DM patients with abnormal eGFR <90 ml/min/1.73 m². The significant differences among groups. It was observed that megalin and uVDBP concentration levels were non-significantly higher in G3 than G1 and G2. It also shows that the level of eGFR in G3 is significantly lower than those in G1 and G2 (P=0.005, P=0.004) respectively, while there was no

Author/Year/Country	Title	Study subjects and methodology	Result (presented as mean \pm SD)	Conclusion
Fawzy and Abu AlSel (2018). Saudi Arabia	Assessment of vitamin D-binding protein and early prediction of nephropathy in type 2 Saudi diabetic patients	<u>Study subjects: 160</u> -G1: 40 healthy volunteers. 120 T2DM patients: -G2: normoalbuminuria. -G3: microalbuminuria. -G4: macroalbuminuria. <u>Methodology:</u> Quantitative sandwich ELISA	uVDBP levels were significantly elevated in G3 and G4 individuals compared with those of the G1 and G2. <u>uVDBP levels (ng/mg):</u> G1=(127.7 \pm 21.9) G2=(193.1 \pm 141.0) G3=(820.4 \pm 402.8) G4=(1458.1 \pm 210.0)	-uVDBP is a good marker for the early detection of renal disease in Saudi T2DM patients.
Khodeir et al. (2016) Egypt	Urinary level of vitamin D-binding protein as a new biomarker for diabetic nephropathy	<u>Study subjects: 60</u> -G1:15 healthy people. 45 Patients with T2DM: -G2: normoalbuminuric. -G3: microalbuminuric. -G4: Macroalbuminuric. <u>Methodology:</u> Quantitative sandwich ELISA	uVDBP levels were significantly elevated in G3 and G4 compared to G2 and G1. <u>uVDBP levels:</u> -G1=(16.400 \pm 4.881) -G2=(20.000 \pm 5.720) -G3=(247.267 \pm 36.654) -G4=(1449.333 \pm 643.456)	-uVDBP levels are significantly increased in patients with DN. -There is a strong positive correlation between the concentration of uVDBP and the development of DN. -uVDBP levels are a potential biomarker for the early detection of DN in T2DM patients.
Shoukry, Bdeer Sel, and El-Sokkary (2015). New York	Urinary monocyte chemoattractant protein-1 and vitamin D-binding protein as biomarkers for early detection of diabetic nephropathy in type 2 diabetes mellitus	<u>Study subjects: 100</u> -G1: 25 healthy individuals. 75 T2DM patients: -G2: 25 normoalbuminuria. -G3: 25 microalbuminuria. -G4: 25 macroalbuminuria. <u>Methodology:</u> Quantitative sandwich ELISA	-uVDBP levels increased significantly in G3 and G4. <u>uVDBP/urinary creatinine levels (ng/mg):</u> G1=(123.4 \pm 28.2) G2=(472.5 \pm 123.6) G3=(984.3 \pm 215.3) G4=(1516.3 \pm 228.6)	-uVDBP could be considered as novel diagnostic biomarker for diabetic nephropathy early detection.
Ali et al. (2015) Baghdad	Estimation of urinary vitamin D binding protein as a biomarker in type 2 diabetic nephropathy and its correlation with estimated glomerular filtration rate	<u>Study subjects: 90</u> -G1: 20 individuals (control group), apparently healthy with no diabetes and kidney diseases and with matched age and sex. -G2: 40 T2DM Patients with normal eGFR (≥ 90 ml/min/1.73 m ²). -G3: 30 T2DM patients with abnormal eGFR (< 90 ml/min/1.73 m ²). <u>Methodology:</u> Quantitative sandwich ELISA	<u>uVDBP levels (ng/ml):</u> G1=(250.5 \pm 0.526) G2=(350.63 \pm 0.8) G3=(503 \pm 14.056) The difference of uVDBP in all between all groups was significant. <u>eGFR (ml/min/1.73 m²):</u> G1=97.16 \pm 1.177 G2=93.91 \pm 1.834 G3=88.13 \pm 1.543 The differences of eGFR in G1 and G3, and G2 and G1 were significant.	-uVDBP levels in all the groups have significantly increased in patients with DN. -There is a negative correlation between the elevated uVDBP levels and decreased eGFR. -uVDBP levels are a novel predictor for the early detection of DN in T2DM patients.
Tian et al. (2013). China	Elevated urinary level of vitamin D-binding protein as a novel biomarker for diabetic nephropathy	<u>Study subjects: 150</u> -G1: 45 healthy volunteers. 105 diabetic patients: -G2: 30 T2DM and 5 T1DM with normoalbuminuria. -G3: 30 T2DM and 5 T1DM with microalbuminuria. -G4: 28 T2DM and 7 T1DM with macroalbuminuria. <u>Methodology:</u> Quantitative sandwich ELISA	uVDBP levels were significantly elevated in G3 and G4 individuals compared with those of the G1 and G2. <u>uVDBP levels (ng/mg):</u> G1=(125.48 \pm 98.27) G2=(466.54 \pm 213.63) G3=(1,011.33 \pm 325.30) G4=(1,406.34 \pm 239.66)	-Patients with DN have significantly elevated uVDBP levels. -There is a strong positive correlation between the concentration of uVDBP and the development of DN. -uVDBP levels are a potential indicator for the early detection and prevention of DN.

Table 4: Shows the previous studies of VDBP as a biomarker in DN.

Author/Year/Country	Title	Study subjects and methodology	Results	Conclusion
Ogasawara et al. (2012) Japan	Significance of urinary full-length and ectodomain forms of megalin in patients with type 2 diabetes.	160 control group with normal eGFR values (60–104 mL/min/1.73 m ²) and urinary albumin creatinine ratio (<10 mg/g). 68 Patients with T2DM. Methodology: Sandwich ELISA for urinary full-length Megalin (COOH (C)-Megalin) and ectodomain (amino (A)-Megalin).	The urinary full-length form of megalin was elevated as DN progresses in T2DM. Patients with normo-albuminuria has significantly high urinary C-megalin levels than control group. The presence of C-Megalin has a better correlation with low eGFR than albuminuria	Urinary full-length and ectodomain form of megalin are biomarkers that indicates the progression of DN in T2DM.

Table 5: Shows the previous study of megalin as a biomarker in DN.

significant difference between G1 and G2, which is expected due to their classification. Moreover, the concentration of Hb1Ac and glucose level were significantly lower in G1 than G2 and G3 (P=0.016, P=0.005), (P=0.011, P=0.006) respectively, in which those tests used in diagnosing DM [11]. Additionally, comparing the duration of T2DM among G2 and G3 did not show any significance. As mentioned previously, the progression of DN is affected by the duration [5], so it was expected to have significant longer duration of T2DM in G3 than G2, while it was shown that the duration of T2DM in G2 is non-significantly longer than G3. This could be due to the small sample size in which each group involve only seven individuals. Moving to the kidney profile tests, only the urea nitrogen test has shown a significant difference between groups. It shows that the concentration of urea nitrogen in G2 is significantly higher than G1 (P=0.006) and no significant differences were found in G3 compared to G2 and G1. However, urea nitrogen values were in the normal range for all the groups. For G1 and G2 it was expected due to their normal eGFR values, but for G3 that has abnormal eGFR, it could be due to the minimal changes in the kidney structure and function that occurred at the early stages of kidney diseases [23], which mainly affects the eGFR in which urea nitrogen could not be affected yet. This also can explain the normal results of uric acid and serum creatinine. However, comparing serum creatinine with eGFR proves the fact that increased serum creatinine will decrease eGFR [11]. Moreover, it showed no significant differences between the studied groups of lipid profile tests that include cholesterol, triglyceride, LDL and HDL as well as liver profiles, which include albumin and total protein. Furthermore, other tests containing homocysteine, insulin, C-peptide of insulin and BMI were measured for the three groups and all of them show non-significant differences. Furthermore, the values of those tests are within the normal reference ranges, except for HDL levels, which appear to be uniformly decreased in G3 compared to G1. Decreasing of HDL level in diabetic patients may associate with CKD. Triglyceride values vary in G3 in which some are normal and others are abnormal in which it usually increases in diabetic patients. However, normal triglyceride level could be referred to a controlled diet. In addition, measurements of serum albumin and total protein are proper tests of liver function because they measure the synthetic and metabolic pathways for these proteins [11]. Regarding insulin and C-peptide of insulin that shows normal ranges, most of the diabetic subjects were taking tablets to control the hyperglycemia that prevents or delay any of the complications. According to correlation analysis was used to figure out the association between different variables. These variables include eGFR, megalin, uVDBP and duration of T2DM. The results revealed that the correlation of eGFR with uVDBP and megalin was negatively weak with non-significant P-value. This proves the fact that low eGFR result in proteinuria including increased secretion of VDBP and megalin [8]. Moreover, it was shown that the correlation between the duration of T2DM with uVDBP and megalin were positive with non-significant P-value. As mentioned previously, the progression of

DN is influenced by the duration of the disease that leads to proteinuria [5]. Furthermore, it was indicated that the correlation between uVDBP with megalin was non-significantly positive. This explains the role of megalin in the endocytosis of uVDBP, where in DM patients, this process is damaged due to proteinuria and increased uVDBP secretions that result in PTECs injury and shedding of megalin in the urine [10]. Previous studies demonstrate that urinary VDBP and megalin are novel potential urinary biomarkers of DN in T2DM patients (Table 4 and Table 5). There were five different studies about uVDBP as a biomarker, where they were conducted in Saudi Arabia [7], Egypt [5], New York [27], Bagdad [26] and China. All of them indicated that uVDBP is considered as a potential biomarker that indicates the early stage of DN in T2DM patients. However Tian et al. [1] had a population of T2DM patients with few patients of T1DM. Furthermore it indicated that there is a strong positive correlation between the concentration of uVDBP and the development of DN. Moreover, demonstrated that there is a negative correlation between the elevated uVDBP levels and decreased eGFR. In addition, there was one study about megalin as a biomarker that was conducted in Japan by [30], which reveal that the urinary full-length and ectodomain form of megalin are biomarkers that indicates the progression of DN in T2DM. The current study demonstrates that the urinary VDBP and megalin levels were non-significantly elevated in T2DM patients with DN. Moreover, a weak negative correlation was observed between eGFR with urinary VDBP and megalin levels [11].

Conclusion

In conclusion, DN is one of the most important complications in T2DM. Previous studies revealed that T2DM patients with nephropathy have higher concentrations of uVDBP that carries vitamin D to the target tissues, and megalin that mediates endocytosis in the proximal tubule than those who are healthy. The aim of the project is to study the urinary VDBP and megalin concentrations as biomarkers in Qatari T2DM patients with low eGFR that indicates the progression of nephropathy. Urinary VDBP and megalin were measured using ELISA. The study subjects were divided into three groups; group 1 (control group with eGFR \geq 90 mL/min/1.73 m²), group 2 (T2DM patients with eGFR \geq 90 mL/min/1.73 m²) and group 3; (T2DM patients with eGFR < 90 mL/min/1.73 m²). This study reveals that urinary VDBP and megalin levels were non-significantly elevated in T2DM patients with DN. Moreover, a weak negative correlation was observed between the urinary VDBP and megalin levels with eGFR. However, it is suggested that these results could be due to some limitations. Further tests and experiments should be performed on larger sample size where the diabetic samples are from a diabetic clinic to confirm the association of megalin and VDBP in T2DM nephropathy.

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Compliance with Ethical Standards

The experimental design including participation of human subjects were obtained from the Qatar Biobank (QBB), under the project no. Ex-2017-QBB-RES-ACC-0082-0029.

Conflict of Interest

The authors declare that they have no conflicts of interest.

Authors' Contribution

Elham Sharif (corresponding author) designed and supervised the project. Naila Kitaz, Amal Hassan, Mariam Alwakeel contributed to data collection, data analysis, and manuscript draft. All authors contributed to the final editing and revision of the manuscript.

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