# Is Serum Vasohibin 1 Level Associated With the Development of Nephropathy in Diabetic Patients?

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**Research article**

**Keywords:** Diabetic nephropathy, Proteinuria, Vascular endothelial growth factor-A, Vasohibin-1

**DOI:** https://doi.org/10.21203/rs.3.rs-67801/v1

# Abstract

**Background:** Glomerular filtration surface area and glomerular filtration rate (GFR) are increased in patients with diabetic nephropathy, especially in the early phase of the disease. Glomerular neoangiogenesis contributes to this increased GFR. Increased GFR is associated with glomerular neoangiogenesis. Vasohibin-1 is an inhibitor of neoangiogenesis. The aim of this study is to evaluate serum vasohibin-1 levels of diabetic patients with and without diabetic nephropathy (DN).

**Methods:** One hundred and five diabetic patients and 39 healthy controls matched for age and gender were included in the study. Diabetic patients with eGFR<60 ml/min/1,73m$^2$ and/or those who had persistent proteinuria (>200 mg/gr) measured by urine protein/creatinine ratio (UP/C) were diagnosed as overt diabetic nephropathy (DN) if they haven't any other known kidney diseases. Serum vasohibin-1 levels were measured by ELISA. Serum creatinine, eGFR, UP/C and vasohibin-1 levels were recorded.

**Results:** Thirty-four percent of diabetic patients had DN. Systolic blood pressure and UP/C were higher in diabetic patients than healthy controls and age, sex, eGFR were similar. Diabetic patients have nonsignificantly lower serum vasohibin-1 levels than healthy controls. Patients with DN had higher UP/C, lower eGFR and long-standing diabetes compared to diabetic patients without nephropathy. Serum vasohibin-1 levels were similar between diabetic patients with and without DN. There was no correlation between vasohibin-1 levels and eGFR or UP/C.

**Conclusions:** In this study for the first time we showed that diabetic patients have slightly lower vasohibin-1 levels than healthy subjects but there was no difference between diabetic patients with and without nephropathy in terms of serum vasohibin levels. Because, vasohibin-1 exhibits its anti-angiogenetic properties by acting via autocrine-paracrine pathways; local vasohibin activity at the tissue level may be more important than the circulating levels.


# Introduction

Diabetic nephropathy (DN) continues to be the most important cause of end-stage kidney disease worldwide [1]. In the early stages of nephropathy, glomerular hypertrophy and increased glomerular filtration rate (GFR) are seen. Proteinuria, mesangial matrix increase and basement membrane thickening are developed over time and eventually led to glomerular sclerosis [2]. Numerous molecules such as Renin-Angiotensin system (RAS), advanced glycosylation end products, fibrogenic transforming growth factor 1 (TGF-1) and insulin-like growth factor 1 (IGF-1) play a role in this process [3, 4].

Neoangiogenesis is observed in nephropathy development as well as retinopathy development among diabetic rats [5, 6]. Animal studies with type 1 and type 2 diabetic rodents revealed an increase in the total length of glomerular capillaries and new glomerular capillary formation [5, 6]. This new vessel formation contributes to increased glomerular surface area and increased glomerular filtration rate in the early stages of nephropathy and hence accelerates the development of nephropathy. VEGF-A, the key molecule of neoangiogenesis, is known to contribute to this process. Overexpression of VEGF-A leads to mesangial

matrix increase and basement membrane thickening in rat kidneys similar to DN [7]. VEGF-A and VEGF-A receptor levels were found to be high in diabetic nephropathy models [8, 9]. Numerous anti-VEGF-A therapies are being tried to treat diabetic nephropathy.

Vasohibine-1 is a peptide produced mainly by endothelial cells and exhibits anti-angiogenetic properties by acting via autocrine-paracrine pathways [10]. It is considered as a negative feedback regulator of angiogenesis [10]. Increasing the vasohibine-1 expression by adenoviral vector transfer in Type 1 and Type 2 diabetic mice, led to an improvement in microalbuminuria, a decrease in increased glomerular filtration rate, and an improvement in pathological changes [11, 12]. However unfortunately, there is little research on the relationship between vasohibine-1 levels and existence of diabetic nephropathy in humans. The aim of this study is to evaluate serum vasohibin-1 levels of diabetic patients with and without nephropathy.

# Material And Methods

This cross-sectional study conducted at nephrology and endocrinology outpatient clinics of Kahramanmaras Sutcu Imam University Faculty of Medicine, Turkey. One hundred and five patients over 18 years old of age, who already followed with the diagnosis of type 1 or type 2 diabetes mellitus and 39 healthy controls were included in the study. Type 1 diabetic patients with a history of diabetes less than 5 years and patients under 18 were excluded from the study. Also the patients who have active malignancy or history of malignancy, an active infection or kidney disease other than related to DM (such as glomerular diseases, polycystic kidney disease, interstitial nephritis, etc.) were excluded from the study because it is known that some malignancies and infections may affect serum Vasohibin-1 levels. Presence of diabetic retinopathy, coronary artery diseases, peripheral artery disease, diabetic neuropathy, Hypertension (HT) and information of the duration of DM, oral antidiabetic or insulin usage were obtained from patient records. Fundoscopic examination of the patients was performed to investigate the presence of diabetic retinopathy by a single ophthalmologist if retinopathy examination was not performed within 1 year. The patients' body weight and height were measured and Body Mass Index (BMI) was calculated by dividing the body weight (kg) of the patients by the square of their height (meters).

Because of the diagnosis of Diabetic Nephropathy (DN) is almost always based on clinical and laboratory findings and exclusion of other possible kidney diseases around the world we diagnosed DN if a patient has type 2 diabetes or longstanding type 1 diabetes for at least 5 years with persistent significant proteinuria and/or low eGFR (< 60 ml/min/1,73 m2). If the patient had findings that impicates the existence of other renal diseases such as rapidly decreasing GFR, rapidly increasing proteinuria, refractory hypertension, active urinary sediment, signs of other systemic diseases and a significant decline in GFR within 2–3 months after starting angiotensin-converting enzyme inhibitor or angiotensin receptor blocker therapy he or she was not determined as DN.

To define diabetic patients with nephropathy we used protein/creatinine values from spot urine sample which were measured from first urine in the morning. We had measure total protein in spot urine since albuminuria measurements were not available in our hospital. Therefore, we compulsorily used urine protein/creatinine ratio (UP/C) instead of urine albumin/creatinin ratio to define significant proteinuria. Although current guidelines use spot albumin /creatinine for the diabetic nephropathy diagnosis; studies show that the results of spot protein/cretinine and spot albumin/creatinine are highly correlated [13]. According to the 2002 chronic kidney disease guideline [14] of KDOQI, protein/creatinine value above 200 mg/gr in a spot urine sample was defined as significant proteinuria. Estimated glomerular filtration rates (eGFR) of patients were calculated using the CKD-EPI formula from serum creatinine values [15]. We retrospectively reviewed the patients' laboratory records for previous UP/C and serum creatinine values to confirm that proteinuria and low eGFR were persistent for more than 3 months.

Patients who were kidney transplant recipients and who had chronic kidney disease other than DN with known ethiology such as any type of acute or chronic glomerulonephritis, polycystic kidney disease, amyloidosis, nephrolithiasis, acute or chronic interstitial nephritis were excluded from the study. For the control group 39 healthy subjects that matched with diabetic patients group in terms of age and gender were recruited from the hospital staff. Fasting blood glucose, HbA1c, serum creatinine (Cr), Na, K, Albumin, Uric Acid (UA), LDL cholesterol, and triglyceride levels of the patients were measured by routine biochemical methods from the blood samples taken at 8:00 a.m. following a fasting period of around 8 hours. Two millilitres of blood samples were taken at the same time to determine the Serum vasohibine-1 level, were centrifuged at 3000 rpm for 5 minutes and the separated serums were stored at -80 C. Serum vasohibine-1 levels were measured by the ELISA method using "Human Vasohibine-1 ELISA Kit "(Lot: 30211832), afterwards all serums were thawed simultaneously at room temperature in accordance with the instructions of the manufacturer. The measuring range of the kit was 0.156-10 ng/mL. The Kit's Intra-assay Precision was ≤ 8%, The Inter-assay Precision was ≤ 12%. Written informed consent was obtained from all patients to participate in the study. Ethical approval was obtained from the Kahramanmaras Sutcu Imam University local ethics committee on 07.11.2018 with resolution number 29 and session number 2018/20.

### Statistical Analysis

The data obtained by measurement were expressed as mean ± SD, and the data obtained by counting as numbers or ratios. Distribution analysis of continuous data were evaluated with Kolmogorov-Smirnov and Shapiro-Wilk tests. In the comparison of the two groups, for the data obtained from measurements, the t-test was used if the data matching the normal distribution, and the Mann Whitney U test was used if the data were not matching the normal distribution. Correlation analysis of continuous data were evaluated with Pearson or Spearman correlation analysis according to the distribution of data. Any P value < 0.05 was considered statistically significant.

# Results

Of the 105 diabetes patients included in the study, 17.1% had Type 1 diabetes and 82.9% had Type 2 diabetes. Forty six percent of DM patients had HT. Seven point six percent of DM patients had neuropathy, 15.2% had retinopathy and 16.2% had coronary artery disease. We diagnosed diabetic nephropathy in 34.3% of all diabetic patients. There is not any significant difference between diabetic patients and healthy controls in terms of age, gender distribution, BMI and eGFR ($p > 0.05$ for all). As expected, systolic blood pressure (SBP) (128.8 ± 16.5 mmHg vs. 123.5 ± 8.7 mmHg $p = 0.01$), urine protein/creatinine ratio (415.9 ± 755.7 mg/gr vs. 102.9 ± 36 mg/gr; $p < 0.001$) and triglyceride values (175.2 ± 90 mg/dl vs 138.5 ± 66.3 mg/dl; $p = 0.03$) of diabetic patients were higher than those of healthy controls, while their albumin (4.2 ± 0.4 gr/L vs. 4.4 ± 0, 2 gr/L; $p = 0.03$) and LDL cholesterol (115 ± 38.3 mg/dl vs 144.3 ± 33.4 mg/dl; $p < 0.001$) levels were lower (Table 1). Although serum vasohibine-1 levels were lower in diabetic patients than in healthy controls, this difference was not statistically significant (321.46 ± 56.48 pg/ml vs 350.1 ± 101.4 pg/ml; $p = 0.1$).

Table 1
Comparison of diabetic and healthy patients

| | DM<br>n = 105 | Healthy<br>n = 39 | p |
|---|---|---|---|
| Age (years) | 50.3 ± 12.1 | 47.2 ± 6 | 0.12 |
| Sex (M)% | 43.8 | 56.4 | 0.2 |
| Type of DM (Type1/Type2)% | 17.1/82.9 | - | |
| DM duration (years) | 9.4 ± 7.4 | - | |
| BMI (kg/m2) | 30.3 ± 7.2 | 29.2 ± 3.8 | 0.2 |
| Hypertension (%) | 46.7% | - | |
| Patient receiving insulin (%) | 55.2 | - | |
| Neuropathy (%) | 7.6 | - | |
| Nephropathy (%) | 34.3 | - | |
| Retinopathy (%) | 15.2 | - | |
| Coronary Disease (%) | 16.2 | - | |
| Urine Protein/creatinine ratio | 415.9 ± 755.7 | 102.9 ± 36 | < 0.001 |
| Systolic Blood Pressure | 128.8 ± 16.5 | 123.5 ± 8.7 | 0.01 |
| Diastolic Blood Pressure | 77.6 ± 10.7 | 78.9 ± 7.0 | 0.4 |
| eGFR | 102 ± 25.4 | 103.7 ± 11.9 | 0.4 |
| Creatinine | 0.8 ± 0.6 | 0.75 ± 0.1 | 0.6 |
| Fasting Blood Glucose | 175.4 ± 65.2 | 93.6 ± 8.2 | < 0.001 |
| HbA1c | 8.5 ± 1.8 | - | |
| Albumin | 4.2 ± 0.4 | 4.4 ± 0.2 | 0.03 |
| Uric acid | 5.0 ± 1.6 | 4.8 ± 1.2 | 0.6 |
| LDL chollesterol | 115 ± 38.3 | 144.3 ± 33.4 | < 0.001 |
| TG | 175.2 ± 90 | 138.5 ± 66.3 | 0.03 |
| Vasohibine 1 | 321.46 ± 56.48 | 350.1 ± 101.4 | 0.1 |

*BMI: Body Mass Index, eGFR: estimated Glomerular filtration rate with CKD-EPI equation, TG: Triglyseride*

Age, sex, BMI, eGFR values, and systolic and diastolic blood pressures of 36 patients with diabetic nephropathy were not significantly different from those without nephropathy ($p > 0.05$ for all). Mean protein excretion of patients with diabetic nephropathy was 956.81 ± 1077.75 mg/g. Serum vasohibine-1 levels in patients with diabetic nephropathy were not different from those without nephropathy (325.55 ± 55.3 pg/ml vs. 319.32 ± 57.3 pg/ml; $p = 0.6$) (Table 2). When the analyzes were repeated excluding type 1 diabetes patients, the results did not change.

Table 2
Comparison of diabetic patients with and without nephropathy

| | DM Nephropathy (-) N = 69 | DM Nephropathy (+) N = 36 | p |
|---|---|---|---|
| Age (years) | 49.8 ± 12.7 | 51.5 ± 11.1 | 0.5 |
| Sex (M)% | 50.7/49.3 | 30.6/69.4 | 0.07 |
| Type of DM (Type1/Type2)% | 17.4/82.6 | 16.7/83.3 | 1.0 |
| DM duration (years) | 8.3 ± 6.5 | 11.5 ± 8.7 | 0.057 |
| BMI (kg/m2) | 29.4 ± 6.4 | 32.0 ± 8.3 | 0.08 |
| Urine Protein/creatinine ratio | 116.45 ± 39.6 | 956.81 ± 1077.75 | < 0.001 |
| Systolic Blood Pressure | 126.8 ± 14.5 | 132.7 ± 19.4 | 0.07 |
| Diastolic Blood Pressure | 77.2 ± 11 | 78.4 ± 10 | 0.5 |
| eGFR | 108.2 ± 15 | 90.3 ± 35.5 | 0.06 |
| Creatinine | 0.66 ± 0.16 | 1.03 ± 1.07 | 0.05 |
| Fasting Blood Glucose | 170.0 ± 71.7 | 185.9 ± 49.6 | 0.01 |
| HbA1c | 8.2 ± 1.6 | 9.0 ± 2.1 | 0.06 |
| Albumin | 4.3 ± 0.2 | 4.0 ± 0.4 | < 0.001 |
| Uric acid | 4.8 ± 1.4 | 5.4 ± 1.8 | 0.09 |
| LDL | 116.2 ± 38.9 | 112.8 ± 37.5 | 0.7 |
| TG | 166.1 ± 91.6 | 192.5 ± 85.4 | 0.08 |
| Vasohibine 1 | 319.32 ± 57.3 | 325.55 ± 55.3 | 0.6 |

No significant correlation was found between the amount of proteinuria and serum vasohibine-1 levels in any of the groups (Table 3). The vasohibine-1 levels only in patients with diabetic nephropathy were moderately inversely correlated with systolic blood pressure ($p = 0.01$ $r = -0.4$). The vasohibine-1 levels in patients with retinopathy were not different from those without retinopathy (data not shown).

Table 3
Correlation of vasohibine 1 and various parameters in all 3 groups

| | **Vasohibin-1** | | | | | |
|---|---|---|---|---|---|---|
| | Diabetic Nephropathy (-) | | Diabetic Nephropathy (+) | | Healthy | |
| | r | p | r | p | r | p |
| Age | 0.1 | 0.1 | 0.01 | 0.9 | 0.05 | 0.7 |
| eGFR | -0.14 | 0.2 | -0.05 | 0.7 | -0.05 | 0.7 |
| UP/C | 0.01 | 0.8 | 0.09 | 0.5 | -0.1 | 0.4 |
| SBP | -0.009 | 0.9 | -0.4 | 0.01 | 0.02 | 0.8 |
| DBP | -0.05 | 0.6 | -0.1 | 0.3 | 0.05 | 0.7 |
| Albumin | | | -0.2 | 0.2 | -0.004 | 0.9 |
| Uric acid | 0.04 | 0.6 | -0.08 | 0.6 | -0.1 | 0.5 |
| HbA1c | -0.04 | 0.7 | 0.1 | 0.4 | - | - |
| BMI | -0.07 | 0.5 | -0.08 | 0.6 | -0.06 | 0.6 |
| SBP: Systolic Blood Pressure, DBP: Diastolic Blood Pressure | | | | | | |

# Discussion

In this study, we demonstrated that the serum vasohibine-1 levels in patients with clinically diagnosed diabetic nephropathy were not different from those diabetic patients without nephropathy and healthy subjects. We also demonstrated that serum vasohibine-1 levels were not associated with clinical or laboratory findings related with nephropathy such as daily protein excretion, glomerular filtration rate and blood pressure.

It is well known that there is an increase in glomerular filtration rate in the early stages of diabetes. In addition to hemodynamic factors, glomerular neoangiogenesis contributes to this increase. Abnormal vascular structures have been described in the glomeruli both of rats and humans with diabetic nephropathy [5, 6, 16–19]. Moreover, in rodents, an increase in glomerular filtration surface area due to slight elongation in new generated and also the existing capillaries has been shown [5–6]. Angiogenesis is a physiological event that is defined as the formation of new vessels from existing vessels and is regulated by the balance between proangiogenic and antiangiogenic factors. The most important proangiogenic agent is VEGF-A. It is known that there is an increase in levels of VEGF-A, and its receptor VEGFR-2, in both humans with diabetic nephropathy and animal experiments [8, 9, 20, 21]. In experimental models, overexpression of VEGF-A has been shown to lead to basal membrane thickening and mesangial expansion similar to diabetic nephropathy [7]. On the other hand, VEGF-A not only

stimulates angiogenesis but also shows an increase in vascular permeability and chemotactic properties for monocytes [22, 23].

Vasohibine-1 is an antiangiogenic factor discovered for the first time during cDNA microarray assays to detect gene upregulation occurring against VEGF-A administration. It is synthesized by endothelial cells and acts as a negative feedback regulator that inhibits angiogenesis. Hence, vasohibine-1 can be expected to antagonize the deleterious effects of VEGF-A in the development of diabetic nephropathy and hence prevent nephropathy. In type 1 diabetic mice, treatment with vasohibine-1 expressing adenoviral vector increased serum vasohibine-1 levels, resulting in significant reduction in pathological changes, such as renal hypertrophy, glomerular hypertrophy and hyperfiltration, albuminuria, type 4 collagen deposition, and mesangial enlargement [11]. Also inflammatory cell infiltration, TGF B1 level and MCP levels decreased. Treatment with vasohibine-1 resulted in a reduction in the number of receptors for high glucose-induced TGF beta, MCP-1 and advanced glycosylation end products in cultured mesangial cells. The same authors obtained similar results in obese type 2 diabetic mice and also demonstrated that treatment with vasohibine-1 reduced VEGF-A expression in podocytes incubated in a high glucose medium [12]. On the other hand, nephropathic changes due to streptozotocin-induced diabetes mellitus, such as diabetes-induced mesangial matrix enlargement, inflammatory infiltration, and albuminuria were more severe in vasohibine-1 gene heterozygous knockout mice than in vasohibine-1 wild-type mice [24]. These findings suggest that vasohibine-1 possesses inhibitory properties against diabetic changes not only through antiangiogenic pathways, but also through direct effect on mesangial cells and inflammatory cascade.

Unfortunately, the relationship between vasohibine-1 and diabetic nephropathy has not been adequately studied in humans. Recently, a unique human study that investigates serum Vasohibin-1 levels in diabetic patients, Ren et al. have shown that urinary albumin/creatinine ratio positively correlated with serum vasohibine-1 levels in 697 type 2 DM patients. They demonstrated in diabetic patients that those with macroalbuminuria had higher levels of vasohibine-1 than those with microalbuminuria, and that those with microalbuminuria had higher levels of vasohibine-1 than healthy individuals [25]. In our study, we could not demonstrate such a relationship between the presence and severity of proteinuria, and serum vasohibine-1 levels. During angiogenesis, vasohibine-1 is secreted from endothelial cells in the termination zone, which inhibits excessive vessel formation. Vasohibine-1 has autocrine and paracrine effects on tissue locally. In our study, the most plausible reason for the lack of correlation between clinical nephropathy parameters and serum vasohibine-1 levels was that the local paracrine effects of vasohibine-1 may be more important than the vasohibine-1 levels in the blood. The systemic and local activity of vasohibine-1 may be different, similar to the difference between systemic and tissue-level RAS activity. Hinamoto et al., evaluated the renal biopsy findings and serum vasohibine-1 levels of 67 patients with chronic kidney disease caused mostly by etiologies other than diabetic nephropathy, and 22 healthy control patients [26]. Their results showed that serum vasohibine-1 levels were inversely correlated with systolic blood pressure, diastolic blood pressure and age, and were not associated with GFR and proteinuria, suggesting that high urine and serum vasohibine-1 levels were associated with poor renal outcomes. In our study, serum vasohibine-1 levels were inversely correlated with systolic blood pressure

in patients with diabetic nephropathy, however, we did not find any significant relationship between vasohibin-1 levels and other parameters such as age, sex, GFR and proteinuria. On the other hand, cisplatin-induced acute renal failure in vasohibine-1 knockout mice has been shown to be more severe than in wild-type mice [27]. It was observed in renal biopsies taken from patients with kidney disease that vasohibine-1 was expressed in a glomerular crescent and interstitial inflammatory cells, as well as in endothelial cells [28]. This finding suggests that vasohibine-1 may be associated with the progression of inflammation and renal damage and that the kidneys respond to cellular stressors with an increase in vasohibine-1 expression.

Our study has some limitations. First, the diagnosis of DM is usually a clinical diagnosis and absence of proteinuria does not mean that there are no histologically nephropathic changes in the kidney. Although studies show that the results of spot protein/cretinine and spot albümin/creatinine are highly correlated, current guidelines use spot albumin /creatinine for the diabetic nephropathy diagnosis. Evaluation of vasohibine-1 activity at tissue level may yield different results. Second, our study was conducted with a relatively small number of patients. We showed that diabetic patients have slightly lower vasohibin-1 levels but it was not statistically significant. This difference may reach more significant magnitude with larger patient numbers. A larger study in which more patients are included is needed.

# Conclusion

In this study, we showed that serum vasohibine-1 levels in diabetic nephropathy patients were not different from healthy individuals and diabetic patients without nephropathy. There are almost no studies on this subject and further studies are needed.

# Declarations

**Ethical approval:** Ethical approval was obtained from the Kahramanmaras Sutcu Imam University local ethics committee on 07.11.2018 with resolution number 29 and session number 2018/20.

**Human and Animal Rights:** “All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.”

**Conflict of interest:** The authors declare that they have no competing interests

**Availability of data and materials:** The datasets used and/or analysed during the current study are available from the corresponding author on reasonable request.

**Informed Consent:** Informed consent was obtained from all individual participants included in the study

**This article has not been submitted for publication elsewhere.**

**Funding:** This study was not funded by any organisation.

### Author Contibutions

Orcun Altunoren: The hypothesis of the study, study design and execution, data collecting, statistical analyse of the study data, writing of the study

Didem Tutuncu Sezal: Data collection, collection of blood samples and serum seperation for measurement of Vasohibin-1 levels, measurement of patients blood pressures, weight and height

Mahmut Egemen Senel: Data collection, collection of blood samples and serum seperation for measurement of Vasohibin-1 levels, measurement of patients blood pressures, weight and height

Muhammed Seyithanoglu: Measurement of serum vasohibin-1 levels from serum samples

Ilyas Ozturk: Data collection, collection of blood samples and serum seperation for measurement of Vasohibin-1 levels, measurement of patients blood pressures, weight and height

Ozkan Gungor, Ertugrul Erken and Murat Sahin: Writing-review & editing

**Acknowledgements:** Not applicable

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