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Study of the reliability of serum vascular endothelial growth factor and its soluble receptor as markers of diabetic nephropathy in type 2 diabetes patients

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Abstract--Background: The levels of circulating angiogenic and anti-angiogenic factors, namely vascular endothelial growth factor-A (VEGF-A) and soluble vascular endothelial growth factor receptor-1 (sVEGFR-1), have been linked to the development of renal dysfunction due to the proliferation of microvasculature within the kidneys of type 2 diabetic (T2DM) patients. The study aims to scrutinize serum levels of VEGF and sVEGFR-1 in a sample of Iraqi diabetic nephropathy patients to support their reliability as markers for the prediction of nephropathy in type 2 diabetes mellitus patients as well as to assess the ACE inhibitor's effect on the levels of these two markers. Method: The ninety participants of this case-control study were split into three groups: thirty-five patients with T2DM and an equal number of patients with DN and the third group involve twenty apparently healthy individuals as the control group. The two diabetic groups have been further divided into four groups according to the ACE inhibitors use. Laboratory measurements involve the glycemic indices, renal function test with serum VEGF-A and sVEGFR-1. Results: the median serum levels of VEGF-A show valuable discrepancies between the three groups (p -value <0.05) but for sVEGFR-1 it doesn't show a notable difference between the DM and the DN groups only(p -value >0.05). The serum levels of the biomarkers show no significant differences between the ACE subgroups (p -value >0.05). Conclusion:

This study concludes that VEGF-A is a good predictor of the disease. While sVEGFR-1 was a poor predictor of diabetic nephropathy but is associated with worsening renal function. The angiotensin-converting enzyme inhibitors may not affect serum VEGF-A and sVEGFR-1 levels.

Keywords---T2DM, Diabetic nephropathy, VEGF-A, sVEGFR-1, ACE inhibitors.

Introduction

Diabetic nephropathy (DN) also termed diabetic kidney disease (DKD) is clinically defined as microalbuminuria associated with a spontaneous decline in glomerular filtration rate (GFR) in a diabetic patient in the absence of other renal disease indicators(1). The pathophysiological mechanisms underlying DN are complex and can partially be explained by that both metabolic and hemodynamic variables combined to stimulate the production of cytokines and growth factors in the glomeruli and tubules which result in activation of the local renin-angiotensin system (RAS), inflammation, reactive oxygen species (ROS), advanced glycation end-products (AGEs), and various vascular growth factors (such as vascular endothelial growth factor-A. (VEGF-A)) dysregulation. Results in increasing glomerular capillary pressure and permeability, proximal tubular cell atrophy, podocytes abnormality, and persistent microalbuminuria might manifest as an obvious clue of DN in most patients. DN eventually leads to renal failure and without proper management end-stage renal disease (ESRD) might develop(2).

Vascular endothelial growth factor-A (VEGF-A) is the first member of the VEGF family (a family of pluripotent cytokines) to be described and subsequently defined as the master regulator of angiogenesis in both physiological and pathological conditions as it's released and expressed by a wide range of cells. At the renal level, it is secreted mainly by glomerular podocytes and to a lesser extent in tubular epithelial cells and is essential for the survival of endothelial cells, podocytes, and mesangial cells(3). In DN chronic hyperglycemia, blood pressure elevation, hypoxia, angiotensin II and insulin-like growth factor 1 are the major causes believed to stimulate excess podocytes VEGF-A levels and its subsequent pathogenicity Also increased oxidative stress in the diabetic environment drives the aberrant cross-talk between VEGF-A and nitric oxide (NO) pathways driving the inception and the advancement of DN(4). As NO is a vasodilator to the afferent arterioles, boosting glomerular blood flow and transferring greater hydrostatic pressure within the glomerulus, allowing albumin to pass through the filtration barrier leading to albuminuria(5). This partially explains how VEGF-A alters glomerular permeability, which contributes to diabetic proteinuria.

The VEGF-A binds to a tyrosine kinase receptors known as vascular endothelial growth factor receptor (VEGFR-1, VEGFR-2, and VEGFR-3) and can also act through other proteins, like neuropilins, integrins, cadherins, and heparan sulfate proteoglycans. VEGF is likely to activate the VEGFR-2 and VEGFR-1 receptors, but the receptors perform slightly distinct functions. Both receptors are glycosylated, but merely the last glycosylated form of VEGFR-2 promotes cell

proliferation(6). Hypoxia has been shown to alter the expression of VEGFR-2 and VEGFR-1, however to a lower extent than VEGF-A itself and this might be triggered indirectly, as VEGF-A alters both receptor types' expression(7).

Alternative splicing of the VEGFR-1 gene results in a soluble version of VEGFR-1 (sVEGFR-1 or fms like tyrosin -1 (sFlt-1)) was spotted in cultivated endothelial cells and has an anti-angiogenic impact. At the renal level, the glomerular endothelium, peritubular capillaries, and, to a lesser extent, mesangial and tubular cells all express sVEGFR-1 which play important role in the regulation of endothelial cell-cell and cell-matrix interactions(8). The sVEGFR-1 is not engaged in vasculogenesis since it has no "mitogenic" effect on endothelial cells but is significantly involved in the migration of endothelial cells, monocytes, macrophages, and hematopoietic stem cells and so is primarily engaged in pathological angiogenesis (tumors, ischemia, inflammation, preeclampsia, etc.). The sVEGFR-1 was thought to control the amount of VEGF-A available for vascular formation by blocking interaction with other tyrosine kinase receptors VEGFR-2 or VEGFR-3. As a result, sVEGFR1 serves as a decoy to modify VEGF-A levels rather than mediating VEGF-induced signal transduction pathways in endothelial cells (9).

The global prevalence of DN and T2DM is increasing continuously, resulting in increased morbidity and mortality, as well as adding significant socioeconomic burdens on worldwide healthcare systems, and alarming healthcare workers and decision-makers to tackle this issue by developing DN prevention, detection, and treatment strategies. As a result, it's critical to look for a novel mechanism that could be targeted to slow disease progression. However, the available indicators (estimated glomerular filtration rate (eGFR) and albuminuria) have several drawbacks and the search for new biomarkers seemed essential to offering effective DN management and prevention. The study aims to investigate serum levels of VEGF (as a potential mediator of diabetic nephropathy and sVEGFR-1 as an antagonist of VEGF), and their relation to blood glucose, kidney function, and ACE inhibitors in a sample of Iraqi diabetic nephropathy patients to support their reliability as markers for prediction of nephropathy in T2DM patients as well as to assess the nephroprotective role of ACE inhibitors in terms of their effect on the levels of VEGF-A and sVEGFR -1.

Subject and Method

This study conducted during the period from November 2021 to February 2022 and included ninety patients of both genders aged 20 to 65 years during their visit to the endocrinologist and nephrologist private clinics in al-Kut city, Wasit government, Iraq. The participants of this case-control study were split into three groups,

- Group 1; control group included 20 apparently healthy subjects,
- Group 2 included 35 patients with T2DM and diabetic nephropathy (DN) and
- Group 3 included 35 T2DM patients and had no DN.

To study the nephroprotective effects of ACE inhibitors in terms of their influence on the levels of the biomarkers, the DM patients in each group were divided into two groups based on whether they were using ACE inhibitors (Lisinopril or Enalapril) or not. On the basis of the 2019 American Diabetes Association (ADA) recommendation, all T2DM patients have been selected by the endocrinologist (10). While DN patients have been diagnosed based on the urine albumin to creatinine ratio (ACR) [ACR > 3 mgmmol] (11) OR the estimated glomerular filtration rate (eGFR) (60 mlmin^{1.73} m²) over a minimum of three months(12) and a DM duration (from diagnosis) of at least 5 years. For at least three months, the patient was on ACE inhibitors. The control group's healthy subjects were chosen randomly (should be of comparable age, sex, and body mass index (BMI) to the two studied patient groups).

Patients with concurrent infection, debilitating illness, endocrinopathies, autoimmune and metabolic disease, pregnant and lactating women, or using concurrent medications that are known to affect serum levels or cause assay misreading (e.g. angiotensin receptor blockers (13), chemotherapy(14), COX-2 inhibitors(15)) are excluded. Patients who provide incomplete information during the questionnaire completion will also be excluded. Blood pressure, BMI were measured and full history was taken utilizing a patient data collecting form, after the patient rested for 5 minutes in a private laboratory. Then, from each participant, an eight-milliliter blood sample was obtained by a vein puncture; two milliliters of the sample were kept in an EDTA tube for HbA1c measurements, while the remaining blood was clotted at room temperature for 5-10 minutes to obtain the serum after centrifugation, has been divided into two parts: one for instant serum creatinine, urea, and random blood glucose enzymatic analysis using cobas® c 111 autoanalyzer. Then the remaining half is kept in an Eppendorf tube and frozen at -20 °C to assess the biomarker level using a sandwich enzyme-linked immunosorbent assay (ELISA) test kit. Random spot urine samples were taken from each participant and utilized immediately for urine Albumin-Creatinine Ratio (ACR) measurements using the Human® Diagnostic "Combilayzer 13" a urine analyzer equipment and "Combina" urine test strip. The modified diet in renal disease (MDRD) equation was used to compute the eGFR (16).

Statistical analyses

Data were managed and analyzed using the statistical package for social science (SPSS) version 25. Continuous variables presented using measures of tendency when applicable. Categorical variables presented as frequency and percentage. Non-parametric tests used to compare the variables that did not follow the normal distribution. Chi-square test used to compare categorical variables; Fisher's exact test used when chi square was inapplicable. The Spearman correlation was applied to appraise the relationship between the biomarkers and the relevant factors. The diagnostic reliability of the biomarkers for predicting nephropathy was measured using the receiver operator curve (ROC). A p-value of less than 0.05 is outlined as statistically significant.

Results

Regarding demographic variables, there were no obvious variations in BMI, gender, systolic and diastolic blood pressure, or living place across the three study groups (P -value >0.05). However, there was a notable difference in age between the control group and the two diabetes patient groups (p -value <0.05). T2DM duration differs significantly between groups, with the DN group having the longest duration. HbA1c, RBS, urea, creatinine, and e GFR serum levels differ significantly across the three groups (p -value < 0.05) As indicated in the table (1)

Table 1
Demographic characteristics

Character	Group 1 (n=20)	Group 2 (n=35)	Group 3 (n=35)	P-value
Gender (female\male)	10\10	20\15	18\17	0.679
Age (year)	50 $\pm$ 13	56 $\pm$ 12	56 $\pm$ 8	0.015 ^{*b}
BMI (kg/m ²)	27.45 $\pm$ 6	29.10 $\pm$ 8	27.30 $\pm$ 6	0.269
Living place (Village\City)	8\12	14\21	12\23	0.862
T2DM duration (year)	-	10.0 $\pm$ 6	13 $\pm$ 5	0.008 ^{*c}
Systolic blood pressure (mmHg)	12.5 $\pm$ 1.9	14.0 $\pm$ 2	14.0 $\pm$ 4	0.018 ^b
Diastolic blood pressure (mmHg)	8 $\pm$ 0.9	8 $\pm$ 1.5	8 $\pm$ 1.0	0.424
RBS (mg/dl)	105.0 $\pm$ 49	227.0 $\pm$ 191	200.0 $\pm$ 102	0.000 ^{*a b}
Glycated hemoglobin (HbA1c) %	4.350 $\pm$ 1.5	8.7 $\pm$ 3.1	7.2 $\pm$ 2.7	0.000 ^{*a b c}
Creatinine (mg/dl)	0.67 $\pm$ 0.18	0.70 $\pm$ 0.19	1.7 $\pm$ 1.4	0.000 ^{*b c}
Urea (mg/dl)	26.0 $\pm$ 10.2	33.4 $\pm$ 10.5	71.3 $\pm$ 67.6	0.000 ^{*a b c}
e GFR (mL/min/1.73 m ²)	103.55 $\pm$ 18.72	89.10 $\pm$ 26.6	38.70 $\pm$ 34.4	0.000 ^{*a b c}

*: significant difference between the three groups, a: significant difference between group1&2, b: significant difference between group1&3, c: significant difference between group2&3,

Figures (1) demonstrate the median serum levels of the biomarkers, and VEGF-A shows a valuable discrepancy between the three groups (p -value <0.05) but for sVEGFR-1 it doesn't show a notable difference between the DM and the DN groups only(p -value > 0.05). The DN group shows the highest levels of VEGF over the DM and the control groups with the DM group showing a higher level than the control group while the sVEGFR-1 level was considerably reduced in the DM and DN groups than the control group.

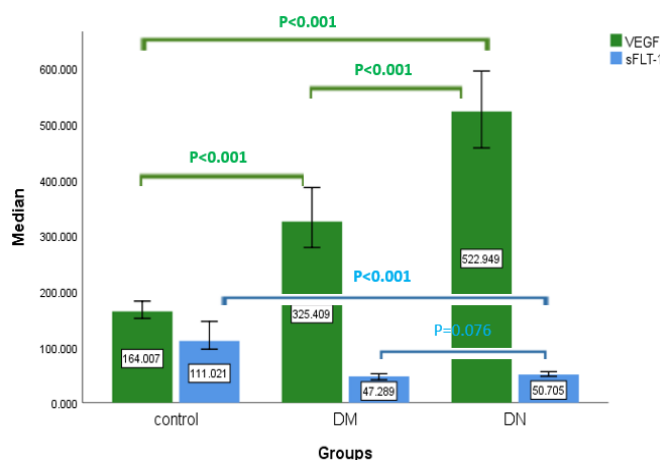


Figure 1. the median serum VEGF-A and sVEGFR-1 level
The serum levels of the biomarkers show no significant differences between the ACE subgroups (p-value >0.05) as shown in table (2)

Table 2
Serum biomarkers levels by the ACE inhibitors using subgroups

Serum biomarker	Type 2 Diabetes mellitus		p. value	Type 2 diabetes with nephropathy		p. value
	With ACE (n=16)	Without ACE (n=19)		With ACE (n=17)	Without ACE (n=18)	
VEGF-A (ng/L) Median ± IQR	282.016 ±89.694	370.175 ±167.053	0.074	485.088 ±119.435	571.632 ±94.411	0.235
sVEGFR-1 (ng/ml) Median ± IQR	43.109 ±15.118	49.506 ±25.108	0.843	49.192 ±14.165	51.3955 ±11.221	0.792

(ACE: angiotensin-converting enzyme inhibitors, VEGF-A: vascular endothelial growth factor-A, sVEGFR-1: soluble vascular endothelial growth factor receptor-1) for assessing the difference significance Man Whitney tests were used.

Table (3) shows Serum VEGF-A has a positive correlation with age, systolic blood pressure, HbA1c, RBS, serum urea, creatinine, ACR, and T2DM duration (p-value<0.05) in addition to a negative correlation with e GFR while there was no correlation between serum VEGF-A and the smoking habit, gender, BMI, and diastolic blood pressure (p-value>0.05). While serum sVEGFR-1 has a negative correlation with BMI, systolic blood pressure, HbA1c, RBS, serum urea, creatinine, ACR, and T2DM duration (p-value<0.05) in addition to a positive correlation with e GFR while there was no correlation between serum sVEGFR-1 and the smoking habit, age, gender, and diastolic blood pressure (p-value>0.05).

Table 3
Correlation of VEGF-A and sVEGFR-1 with different study variables

Variable	VEGF-A		sVEGFR-1	
	r	p-value	r	p-value

Age	0.307**	0.003	-0.151	0.157
Gender	0.29	0.788	-0.058	0.590
Smoking	0.36	0.737	-0.092	0.391
BMI	0.005	0.965	-0.218*	0.039
S B\ P	0.268*	0.011	-0.242*	0.022
D B\ P	0.74	0.488	-0.012	0.912
HbA1c	0.407**	0.002	-0.495**	0.000
RBS	0.458**	0.000	-0.322**	0.002
Urea	0.543**	0.000	-0.297**	0.004
Creatinine	0.540**	0.000	-0.229*	0.030
eGFR	-0.652**	0.000	0.305**	0.003
ACR	0.553**	0.000	-0.276**	0.008
T2DM duration	0.518**	0.000	-0.508**	0.000

RBS: random blood sugar, eGFR: estimated glomerular filtration rate,
 * p-value <0.05, ** p-value < 0.01, r: Spearman correlation coefficient

Serum VEGF-A shows good diagnostic reliability for the prediction of diabetic kidney disease in diabetic patients while sVEGFR-1 shows a poor prediction ability as shown in figures (2) VEGF-A has a higher sensitivity and specificity at the chosen optimal cut-off point while sVEGFR-1 has the lowest values.

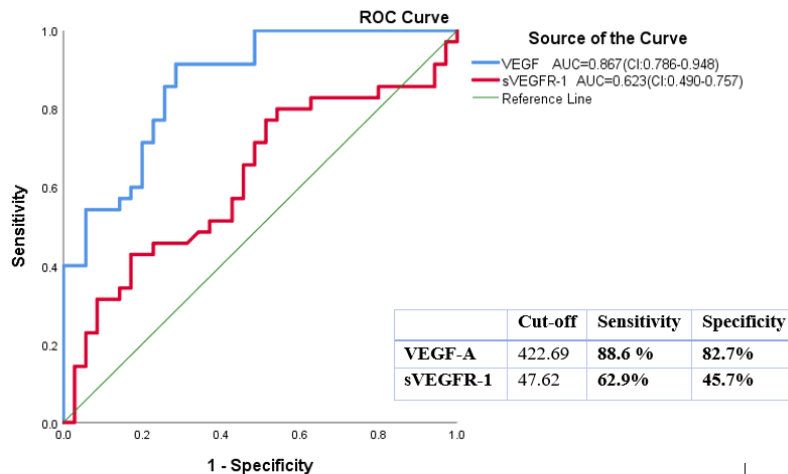


Figure 2. diagnostic reliability of VEGF-A and sVEGFR-1 for the prediction of diabetic kidney diseases in diabetic patients using the ROC curve. (AUC: area under the curve, CI: confidence interval, VEGF-A: vascular endothelial growth factor-A, sVEGFR-1: soluble vascular endothelial growth factor receptor-1)

Discussion

The identification of new novel markers with contrast to microalbumin in urine for the early detection of nephropathy in diabetic patients would allow for early intervention to lessen the effects of long-term vascular complications such as renal impairment and cardiovascular disease(17–19). The levels of circulating angiogenic and anti-angiogenic factors, namely VEGF-A and sVEGFR-1 receptors, have been linked to the development of renal dysfunction due to the

proliferation of microvasculature within the kidney(20). In the present study when diabetic patients with and without nephropathy were compared to healthy controls, serum levels of VEGF were considerably higher. These findings are consistent with the findings of other clinical studies(21–23). The possible explanation is that chronic hyperglycemia and the associated hypoxia lead to increased oxidative stress, inflammation, and angiotensin II to promote VEGF-A production and release(3,24). Another possible mechanism is the unique theory of “uncoupling of VEGF-A with nitric oxide (NO)”.

Endothelial NO generation is normally stimulated by VEGF-A, and NO is necessary for VEGF activities on endothelial cells. When hyperglycemia inhibits normal endothelial function and lowers NO release leads to high levels of glomerular VEGF- A, resulting in diabetic glomerulopathy (5). This reveals that serum VEGF-A is a valid biomarker for assessing the incident and advancement of DN. Veron et al.(25), on the other hand, suggested that a "normal" amount of VEGF-A is required for preserving the glomerular capillary structure in adult kidneys and that both too high and too low VEGF-A in glomeruli can cause severe renal disease. A significant positive correlation between HbA1c, RBS, and VEGF-A has been demonstrated. These results in coordinated with Hanefeld et al.(26) and Zhou et al.(27) who measured serum VEGF-A levels in 345 newly diagnosed type 2 diabetic Chinese patients and found a strong association between VEGF levels and glucose control.

This study found a valuable correlation between serum urea and creatinine, ACR, and eGFR with elevated serum VEGF levels in the DN group this connection may predict chronic kidney disease development and mortality and shows that VEGF, which might be a detectable marker of renal damage before albuminuria develops. Aly et al.(28) and Martynov et al.(29) show that there is a link between higher VEGF levels and albuminuria in diabetic individuals, as well as a decrease in glomerular filtration rate. The serum VEGF level was shown to be favorably connected with urine albumin creatinine ratio and systolic blood pressure but negatively correlated with glomerular filtration rate in a study conducted by KIM et al. (4) which goes in line with the results of the current study. The currently observed positive correlation between diabetes duration and serum VEGF levels probably explains why disease duration is linked to both macro and microvascular events. This is due to the fact that an increase in disease duration combined with poor glycemic control promotes the release of inflammatory mediators and proangiogenic factors like VEGF, which can lead to complications in diabetics (30).

The current study found a substantial decrease in sVEGFR-1 serum levels in both diabetic groups compared to the control group, this might be related to chronic hyperglycemia leading to hypoxia and inflammatory cytokines liberation. On the other hand, this might be explained by the simultaneous dramatic rise in VEGF serum level, which overwhelms the sVEGFR-1 level and lowers its free circulating level, thereby limiting its biological influence(31). There were numerous mechanisms explaining the decreased sVEGFR-1 synthesis, storage, and release from endothelial cells in DN including reactive oxygen species generation, advanced glycation end product, increased renin-angiotensin-aldosterone system activity, and accumulation of an endogenous inhibitor of nitric oxide synthesis,

might all play a role in the worsening renal diseases mechanism by affecting sVEGFR-1(32).

The current results are reflected to those of other studies of T2DM patients that found no significant variabilities in serum s VEGFR-1 levels between diabetic patients with normal renal functions and diabetic nephropathy(4,33) and found a significant negative correlation between serum s VEGFR-1 and other risk factors for diabetic microvascular complications, such as glycemic control, renal function tests (but not eGFR), duration of DM, and BMI. The interpretation of sVEGFR-1 levels in diabetic nephropathy patients is somewhat complicated. In 2009, Di Marco et al.(34) and Onoue et al.(35) published their findings on sVEGFR-1 levels in DN patients that were mutually inconsistent. In patients with CKD who had cardiac catheterization, Di Marco et al. (34) found a negative association between sVEGFR-1 levels and eGFR, whilst Onoue found a positive link, which is consistent with the current study findings. Matsui et al. (36) proposed that heparin administration just before blood sampling for sVEGFR-1 measurement as a possible explanation for the discrepancy in the correlation between sVEGFR-1 levels and eGFR and suggested that sVEGFR-1 was bound to heparin sulfate on the endothelial cell surface had heparin-binding domains.

The link between sVEGFR-1 and its ligand, VEGF, must be considered when interpreting the impact of reduced serum levels of sVEGFR-1 in DN. In this study, serum VEGF levels increased significantly when eGFR decreased whereas serum sVEGFR-1 levels were reduced with eGFR reduction this has been linked to the augmented pathological mechanism that damages the kidney vasculature. Taken together, these findings show that sVEGFR-1 therapy may be advantageous in diabetic nephropathy patients. According to Bus et al. (37), normalizing VEGF-A levels using sVEGFR-1 may be a feasible technique for treating diabetic nephropathy patients and so reversing kidney damage. Despite it's still conceivable that the renin-angiotensin system's activity influences VEGF renal expression and urine excretion, there was no discrepancy in serum VEGF levels between treated and untreated individuals within the two diabetic groups involved in this study. This unanticipated observation is somewhat difficult to explain. It's possible that ACE inhibitor administration prevented a more significant rise in blood VEGF concentrations in patients with advanced renal impairment. On the other hand, this data might simply reflect that patients with higher creatinine clearance had a superior renal clearance of VEGF. Lenz et al. (38) and Chaturvedi et al(39)., also found that circulating VEGF levels were unaffected by ACE inhibition. Also, the lack of association between ACE inhibition and circulating serum s VEGFR-1 in this study might suggest that hat circulating sVEGFR-1 isn't the primary mediator of their effects. The lack of previous research regarding the effect of ACE inhibitors on the sVEGFR-1 level emerges the need for further investigations.

According to the ROC curve, VEGF-A has a high good sensitivity for diagnosing DN in diabetic patients this is in line with Aly et al.(28) findings while S VEGFR-1 exhibited the lowest sensitivity and specificity. Thus, this high sensitivity and specificity of VEGF-A indicate the importance of this parameter as a predictor of metabolic disturbances which promote diabetic vascular complications. The limited sample size and the actuality that the study was carried at a single facility

with participants from just one ethnic group, the findings may not be typical of type 2 diabetes patients globally. Also, all of the measurements are taken at the same time as a result, a causal link cannot be established. Even though it has been observed that serum levels of VEGF are higher than plasma levels as a result of the platelet-derived VEGF released during platelet aggregation, serum levels were measured because certain readings from plasma VEGF levels were lesser than the sensitivity of the ELISA kits as specified by the manufacturer, and thus will not provide any meaningful analysis.

Conclusion

This study concludes that VEGF-A is linked to the progression of DN and is a good predictor of the disease. While sVEGFR-1 was a poor predictor of diabetic nephropathy but is associated with worsening renal function, adding an additional insight to the understanding of sVEGFR-1 as a biomarker for diabetic complications such as nephropathy. The angiotensin-converting enzyme inhibitors may already include peritubular micro-vessel protection but not affect serum VEGF-A and sVEGFR-1 levels as there was any significant correlation.

Recommendations

Investigation of the relationship between these indicators and their performance in various stages of diabetic nephropathy with a larger sample size in a multicenter trial is required. serum VEGF-A is to be used as a diagnostic tool for diabetic nephropathy prediction and research VEGF-A as a therapeutic target in preventing and treating diabetic nephropathy. More study into linkages with ACE, Ang II, VEGF-A, and sVEGFR-1 would aid in a better understanding of the regulatory roles of the pathophysiological processes of DN.

Conflict of Interest: Authors declared none

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Ethical approval

Ethical approval. The ethics committee of the University of Baghdad, College of Pharmacy authorized the study and each individual supplied their informed permission before participating in the study. Data collected in accordance with the World Medical Association, Declaration of Helsinki, 2013 for the ethical principles for medical researches involving human.

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