Original Article

Study of Angiopoietin-2 and vascular endothelial growth factor as markers of diabetic nephropathy onset in Egyptians diabetic patients with non-albuminuric state

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A R T I C L E   I N F O

A B S T R A C T

Background: Vascular endothelial growth factor (VEGF) and angiopoietin (Ang-2) systems have a central role in vasculogenesis and neoangiogenesis during glomerular development. disruption, their levels are associated with alterations in the glomerular filtration barrier and proteinuria as in diabetic nephropathy. Aim of this study to assess the validity of blood Ang-2 and VEGF as biomarkers for early detection of diabetic nephropathy as well as to study the relation between them and inflammation in diabetic nephropathy patients.
Subjects and methods: Cross-sectional study included 180 diabetic nephropathy patients. Patients were classified to non-albuminuric, microalbuminuria and macroalbuminuria patients. Patients with macro-albuminuria complicated to renal impairment and ESRD on top of diabetic nephropathy. Ang-2 and VEGF were measured beside urinary albumin creatinine ratio (UACR).
Results: Significant increase of Ang-2 and VEGF levels among patients with normoalbuminuric state compared to control but there is no significant difference of UACR between both groups. Ang-2 and VEGF concentrations were significantly higher in patients with microalbuminuria and macroalbuminuria compared to healthy. Ang-2 and VEGF levels increase with the progression of albuminuria. There were significant positive correlation between CRP and Ang-2 in addition to VEGF. Significant negative correlation between eGFR, Ang-2 and VEGF.
Conclusion: VEGF and Ang-2 were significantly elevated in diabetic nephropathy patients without albuminuria, their levels steadily increase with the progress of albuminuria, So can use them as markers for diagnosis of diabetic nephropathy onset and progression specially in patients without an increase in albumin excretion.

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1. Introduction

Diabetic nephropathy (DN) is a main microvascular complication of diabetes mellitus, most cases are asymptomatic. delayed the pathological manifestation of glomerular disease in diabetic patients will possibly reverse. Therefore, the early diagnosis and intervention are crucial [1,2]. Many patients with diabetic nephropathy progress to chronic kidney disease (CKD) and end-stage renal disease (ESRD) [3]. Albuminuria is indicative of endothelial damage and vascular disease of renal glomeruli in diabetic nephropathy. Many studies have shown that albuminuria is an independent predictor of cardiovascular events in both CKD and non-CKD patients [4,5]. Moreover, systemic microinflammation also supposes the increased cardiovascular morbidity and mortality [6,7]. Although albuminuria and microinflammation explain the complex interplay in CKD, the effectors mediating the cross talk between CKD and CVD are still not confirmative.Angiogenesis is regulated by circulating angiogenic growth factors, which had trophic and proliferative effects on micro vessels [8] . Vascular endothelial growth factor is protein produced by cells that stimulates vasculogenesis and angiogenesis.Overexpression of it can cause vascular disease in the retina and other parts of the body [9]

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.Ang-2 act as signalling molecule involved in endothelial cells activation and the initiation of angiogenesis [8]. Increased blood levels of Ang-2 has been shown in many systemic inflammatory diseases as diabetes mellitus, chronic kidney and end-stage renal diseases (ESRD) [9]. VEGF and Ang-1 have autocrine/paracrine activity as they bind to their receptors expressed in both podocytes and glomerular endothelial cells after secretion by podocytes. In diabetes an upregulation in VEGF is paralleled by excess in Ang-2 that displaces the Ang-1 binding from the Tie-2 receptor [10] .

Aim of this study to evaluate plasma VEGF-A and angiopoietin as novel biomarkers for early detection of diabetic nephropathy and to determine the relation between them and inflammation in diabetic nephropathy patients.

2. Subjects and methods

Cross-sectional study on 180 type 2 diabetic patients diagnosed according to WHO criteria (100 male, 80 female) follow up in the internal medicine Zagazig University Hospitals, Egypt from April 2015 to May 2017 in addition to 40 healthy individuals as control the study was approved by our University Hospitals institutional ethical committee; written informed consent was obtained from the participants. Patients were enrolled when they met the criteria of WHO (i.e., symptoms of hyperglycemia, random blood glucose ≥ 200 mg/dL, fasting and postprandial blood glucose levels were ≥ 126 mg/dL, ≥ 200 mg/dL respectively, or A1C $\geq 6.5\%$), age not less than 18 years. Any patient had congestive heart failure, hypertension, peripheral artery disease, critical illness, pregnant females, glomerulonephrites and malignancy were excluded. All patients were classified into diabetic patients without micro-albuminuria, microalbuminuria, macroalbuminuria, renal impairment and end stage renal disease (ESRD) patients on top of diabetic nephropathy.

2.1. Samples collection

five ml blood were withdrawn and delivered into plain and EDTA vacutainer tubes for Ang-2 and Vascular endothelial growth factor (VEGF) level estimation in addition to routine investigations as C-reactive protein and kidney function tests. Urine samples were collected for determination urinary albumin and creatinine, the UACR, in mg albumin per gram creatinine was calculated by dividing value of urinary albumin by the urinary creatinine concentration. Microalbuminuria and macroalbuminuria were defined if UACR was 30−300 and > 300 mg albumin/gm creatinine, respectively [11]. The estimated glomerular filtration rate (eGFR) was calculate by Chronic Kidney Disease Epidemiology Collaboration equation [12], . Ang-2 and VEGF assay: Blood Ang-2 and VEGF concentrations were assayed by enzyme-linked immunoadsorbent assay (ELISA) according to the manufacturer's protocol using Human Angiopoietin Π and Human vascular endothelial growth factor

ELISA Kits, Bioneovan Co, Ltd. CRP and albumin in urine were determined by immunoturbidometric assay on cobas c 311/501 analyzers, Roche Diagnostics GmbH, D68298 Mannheim-Germany.

3. Statistical analysis

All data were analyzed using Statistical Package for the Social Sciences (SPSS version 20.0) software for analysis. According to the type of data qualitative represent as number and percentage, quantitative continues group represent by mean ± SD,. Differences between parametric quantitative multiple parametric by ANOVA, non parametric Kruskal Wallis, followed by Post hoc test was done. P value was set at <0.05 for significant results & <0.001 for high significant result.

4. Results

The number of participants in this study were 220 subjects among them 120 males and 100 females, with mean age of 56.8 ± 8.5 years. There are statistical significant difference among groups as regard Ang-2, VEGF, CRP concentrations and UACR, (Table 1). The Ang-2 and VEGF levels among diabetic patients without micro-albuminuria were significantly increased compared to healthy individuals but there is no significant difference of UACR. No significant difference between diabetic patients with or without micro-albuminuria as regard VEGF and Ang-2 levels. Blood VEGF concentrations were increased in macro-albuminuria groups than patients with micro-albuminuria but there was no significant changes of Ang-2 levels between both groups (Table 2),. There were positive correlation between plasma VEGF and Ang-2 levels, there are correlation between them and MAP, CRP and HbA1C. Micro-albuminuria has positive correlation with VEGF levels but not between it and Ang-2 levels. . There are significant negative correlation between eGFR., VEGF and Ang-2 levels. No significant correlation between VEGF levels and age, levels of Ang-2 with age, micro-albuminuria and UACR (Table 3),. VEGF and Ang-2 have high sensitivity and specificity in prediction of diabetic nephropathy at different cutoff values among studied groups (Table 4), Fig. 1.

5. Discussion

The recognition of novel markers in addition to microalbumin in urine for early diagnosis of nephropathy in diabetic patients would facilitate early intervention to reduce the effect of the chronic vascular complications as renal damage and cardiovascular morbidity [13]. The development of diabetic kidney disease due to proliferation of micro vessels of kidney have been demonstrated to associate with increased of circulating angiogenic growth factors levels, among these are angiogenic and anti-angiogenic factors, namely VEGF, VEGF receptors, angiopoietins and endostatin, with respect to therapeutic prevention, implementation of treatment at

Table 1
Comparison between the studied groups as regard blood Ang-2, VEGF and UACR.

	CRP mg/l	eGFR ml/min/1.73 m^2	UACR mg/gm creatinine	Ang-2 pg/ml	VEGF pg/ml
Control (n = 40)	4.8 ± 0.7	101 ± 15	13.2 ± 6.1	51.9 ± 22.3	127.6 ± 40.7
Without micro-albuminuria (n = 35)	9 ± 2.6	80 ± 16	17.5 ± 5.8	131 ± 20.7	341 ± 26.9
Micro-albuminuria (n = 38)	8.8 ± 2.6	75.9 ± 27	141 ± 90.9	155.6 ± 11.8	378 ± 18.9
Macro-albuminuria (n = 35)	13.6 ± 9.8	67.5 ± 22.1	2699 ± 224.7	189.4 ± 9.5	438 ± 33.8
Renal impairment (n = 38)	11.8 ± 6	43.2 ± 8.3	2128 ± 204.7	280.3 ± 41.4	606 ± 28.6
ESRD (n = 34)	16 ± 7.1	15.9 ± 7.7	4636 ± 204.1	473.2 ± 63	668 ± 33.8
F	3.2	13.5	28.6	270.7	484.9
P	<0.01	<0.01	<0.001	<0.001	<0.001

Ang-2:Angiopoietin-2, VEGF:Vascular endothelial growth factor, UACR: Urinary albumin-creatinine ratio, CRP: C-reactive protein, eGFR: estimated glomerular filtration rate ESRD:end stage renal disease.

Table 2
LSD Post-hoc test for Ang-2, VEGF levels, UACR and CRP among different groups.

Group	Mean ± SD
Control	DM è micro-albuminuria[a*,b*,c], Micro-albuminuria[a**,b**,c**], Macro-albuminuria[a**,b**,c**d**] Renal impairment[a**,b**,c**], d* ESRD[a**,b**,c**,d**]
Without micro-albuminuria	Micro-albuminuria[b,c**], Macro-albuminuria[a*,b*,c**] Renal impairment[a**,b**,c**], d* ESRD[a**,b**,c**], d*
Micro-albuminuria	Macro-albuminuria[b*,c**, d] Renal impairment[a**,b**,c**], d* ESRD[a**,b**,c**], d*
Macro-albuminuria	Renal impairment[a**,b**,c**], d* ESRD[a**,b**,c**], d*
Renal impairment	ESRD[a**,b*,c**], d*

*Significant P < 0.05, **Highly significant P < 0.001.
a: LSD of plasma Ang-2 level among different groups.
b: LSD of plasma VEGF level among different groups.
c LSD of plasma UACR among different groups.
d: LSD of plasma CRP among different groups.

Table 3
Correlation between blood VEGF and Ang-2 with Lab parameters.

	VEGF (pg/ml)		Ang-2 (pg/ml)	
	r	p	r	p
Age (year)	0.013	0.901	0.122	0.253
MAP (mmHg)	0.482	0.001	0.696	0.001
Serum creatinine (mg/dl)	0.629	0.001	0.800	0.001
eGFR (ml/min/1.73 m^2)	- 0.624	0.001	- 0.708	0.001
CRP (mg/l)	0.478	0.04	0.443	0.05
HbA1C %	0.729	0.002	0.543	0.036
Microalbuminuria mg/dl	0.363	0.05	0.177	0.096
UACR (mg albumin/gm creatinine)	0.426	0.05	0.182	0.086
VEGF (pg/ml)			0.857	0.001

Ang-2 = Angiopoietin-2, VEGF = Vascular endothelial growth factor, CRP = C-reactive protein, HbA1C = glycated hemoglobin, ACR = albumin-creatinine ratio, ESRD = end stage renal disease, MAP = mean arterial pressure,.

early stage of diabetic and nondiabetic kidney disease is able to restore renal perfusion and function [14]. This cross-sectional study in the period from March 2015 to May 2017 to evaluate plasma VEGF and Ang-2 as new biomarkers for early detection of diabetic nephropathy. In the present study, plasma levels of Ang-2 and VEGF were significantly higher in patients with and without micro-albuminuria as well as macroalbuminuria compared to healthy subjects. Ang-2 and VEGF levels steadily increases with the progression of albuminuria and renal impairment. The current study showed significant positive correlation of both VEGF and Ang-2 levels with MAP, CRP and HbA1c as well as VEGF only correlated with micro albumin and urinary albumin creatinine ratio not Ang-2. No significant correlation of age with changes of VEGF and Ang-2

levels, Significant negative correlation between eGFR with VEGF and Ang-2 levels, VEGF and Ang-2 levels are increased in parallel to poor glycemic control as reflected by their correlation with HbA1c. and their association with changes in CRP levels, this implies that elevated levels of these biomarker occur as a result of inflammation and vascular dysfunction as a part of atherosclerotic process. Chen et al., found no significant difference in blood levels of VEGF among diabetic patients compared to healthy individuals however, urinary VEGF levels of patients higher than the control subjects. Blood levels of Ang-2 were significantly positively correlated with urinary Ang-2 and VEGF levels [15]. Another study conducted by Lip et al., showed that in the patients and controls, plasma VEGF levels were significantly correlated with plasma Ang-2 among diabetic patients only [16]. Previous studies of plasma angiopoietin (Ang-1 &2) and VEGF in type-2 diabetic patients with and without chronic vascular diseases, demonstrated the elevation of VEGF and Ang-2 (but not Ang-1) in diabetic patients compared to healthy individuals with no significant difference between patients with and without cardiovascular disease [17]. Accumulation of advanced glycation end-products (AGE) under effect of hyperglycemia prompts VEGF production by mesangial cells and can up-regulate VEGF and Ang-2 mRNA expression [18,19]. VEGF is a potent stimulus of Ang-2 release and expression of Ang-2 mRNA [20]. The close relationship between these elements, suggesting a tight and preferential control mechanism for angiogenesis and vascular permeability [17]. The reduction of GFR and progression of chronic kidney disease are related to increase in circulating blood Ang-2 levels which stimulated by increased expression of VEGF, these are essential regulators of angiogenesis [21–23]. This inverse relationship between increased angiogenic growth factors levels and reduction of

Table 4
Validity of plasma ANG-2 and VEGF as predictors of diabetic nephropathy among patients groups.

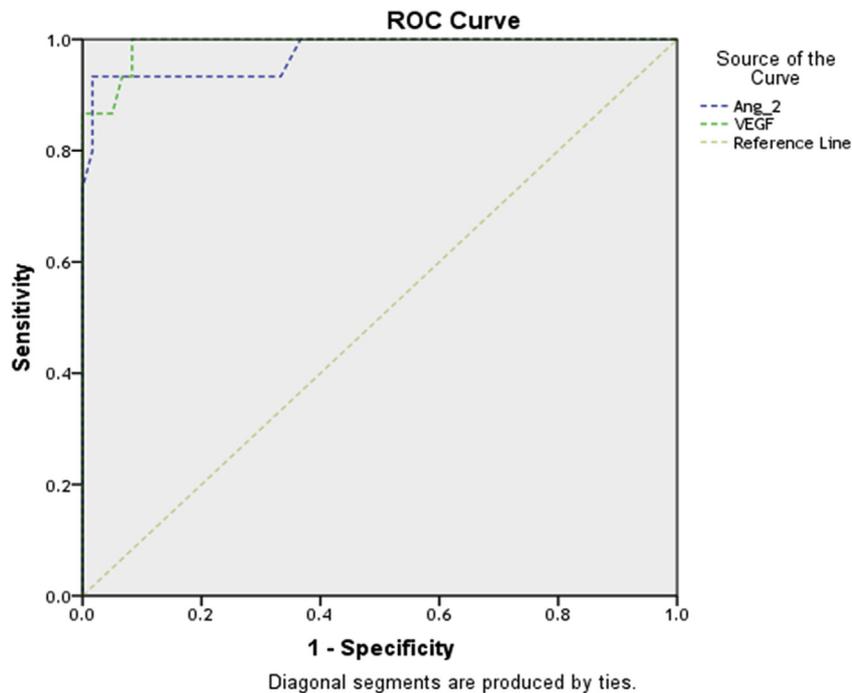|	Ang-2 (pg/ml)			VEGF (pg/ml)		
	Cut off	Sensitivity	Specificity	Cut off	Sensitivity	Specificity
DM è micro-albuminuria	>85	95%	92.5%	>168.3	92.9%	91.1%
Micro-albuminuria	>115	86.6%	93.3%	>272.5	93.3%	100%
Macro-albuminuria	>173	92.9%	96.7%	>398	93.3%	93.3%
Renal impairment	>244	93.3%	98.3%	>550	93.3%	91.7%
ESRD	>365	92.9%	93.3%	>617	98.7%	93.3%

Fig. 1. ROC curve for detection of nephropathy diabetic patients with non albuminiric state using Ang-2 and VEGF levels.

GFR may predict mortality in patients with CKD [24]. Ang-2 levels were markedly elevated in diabetic patients compared with values in the control group, its levels are increased in blood and urine of normoalbuminuric patients. Ang-2 concentrations were significantly higher in patients with macroalbuminuria than patients with microalbuminuria. This observation is likely due to the tubular pathophysiological changes, which is developed before the glomerular stage of disease. This suggests that the subclinical tubular impairment lead to increase in angiogenic growth factors as VEGF and Ang-2 levels these may be an earlier measurable markers of renal involvement before the onset of albuminuria [15] . Rizkalla et al., they demonstrated decreased in renal Ang-1 level and increase of plasma Ang-2 at 8 weeks after the induction of diabetes in a rat model and Ang-2 was observed in endothelia and podocytes of glomeruli [25]. Study was done by Lim et al., found values of VEGF and Ang-2 significantly correlated with HbA1c while there is no significant relationships of change in plasma VEGF, Ang-1 and Ang-2 levels with age [17] . There are relation between increased in VEGF and Ang-2 concentration with increased of albuminuria in diabetic patients as well as reduction of glomerular filtration rate and anemia [26] . Chen et al., found, no significant correlations were found between serum or urinary Ang-2 and age, HbA1c, blood pressure, in addition to lipid profile [15] .The differences between studies may reflect the different populations being studied, as well as variations in study design. The study done by KIM et al., identified the urinary albumin creatinine ratio was positively correlated with the urinary VEGF level, systolic blood pressure and to be negatively correlated with the glomerular filtration rate [27] . Normally Vascular endothelial growth factor (VEGF) is an important regulator of angiogenesis. VEGF regulates angiogenesis as its effects on proliferation and differentiation of endothelial cell, mediates permeability and blood vessels vasodilation but in some pathological conditions of kidney as in diabetic nephropathy increased expression of VEGF in glomeruli causes the glomerular hypertrophy that is associated with proteinuria [28]. Rizkalla et al., they demonstrated decreased in renal Ang-1 level and increase of plasma Ang-2 at 8 weeks after the induction of diabetes in a rat

model and Ang-2 was observed in endothelia and podocytes of glomeruli [25]. There are relationship between the plasma Ang-2 and hsCRP levels [24]. Inflammatory responses of endothelial cell regulated by Ang-2 and it acts as a switch of vascular responsiveness exerting a permissive role for the actions of proinflammatory cytokines [19,29]. Ang-2 serves the link between angiogenic and inflammatory pathway, Ang-2 has a role in renal fibrosis as it signalling between cellular elements of fibrosis, including endothelial cells, pericytes, myofibroblasts, and macrophages [30]. Insulin Resistance Atherosclerosis Study reported the relation between presence of microalbuminuria and elevation of CRP this relation may arise from development of atherosclerosis, effects of cytokines or effect of both on the glomerulus [31] . Two other studies identified the elevation of plasma Ang-2 levels in patients on dialysis compared with healthy controls [22,32]. In one of these studies, plasma Ang-2 correlated with coronary and peripheral arterial disease scoring [32]. Another study showed correlation between plasma Ang-2 with cholesterol, high-sensitive C-reactive protein at same time it was an independent predictor of mortality [22] . Shroff et al., 2013, observed that Ang-2 levels had no significant relation to age or gender, Ang-2 levels were not significantly correlated with blood cholesterol or urinary albumin/creatinine levels in predialysis CKD or dialysis patients [33]. This study showed the association between increased of Ang-2 and VEGF levels and progress of nephropathy as these markers have high specificity and sensitivity in diagnosing of different stages of nephropathy before and after the development of microalbuminuria. Laboratory markers play an important role in the early detection of nephropathy the best known was microalbuminuria. The microalbuminuria represents a marker of the generalized endothelial dysfunction present in DM, linking renal involvement with cardiovascular and cerebral impairment [34] . Albuminuria has been considered the golden standard diagnostic and prognostic biomarker for diabetic nephropathy onset and progression [35], but albuminuria lacks specificity for diagnosing disease progression when the urinary albumin excretion is < 300 mg/24 h and also lacks sensitivity since diabetic nephropathy can frequently progress without an increase

in albumin excretion. Therefore, it does not assist as an accurate surrogate endpoint for the progression of diabetic kidney disease [36]. Albuminuria has been considered assumed as the earliest sign for diabetic nephropathy; however, many cases of renal impairment occurs without microalbuminuria so it is essential to implement different methods for earlier detection of renal impairment in diabetic patients targeting to prevent the long term devastating effects of renal loss in diabetics [37]. Our study demonstrated the elevation of VEGF and Angiopoietin-2 blood levels in patients with diabetic nephropathy. The highest levels of them among patients with complications as renal impairment and end stage renal disease also their levels steadily increases with the progression of albuminuria, suggesting their possible roles as early markers of microvascular complications.

6. Conclusion

VEGF and Ang-2 were significantly elevated in diabetic patients without albuminuria compared with non-diabetic control individuals and could be used as markers of nephropathy at a very early stage even in normoalbuminuric diabetic patients and complicated with renal failure.

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