Serum Levels of Angiotensin II with Type 1 and Type 2 Diabetes Mellitus and Diabetic Nephropathy Patients

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Abstract
Diabetic nephropathy (DN) is the leading cause of end-stage renal disease worldwide. Chronic hyperglycemia and high blood pressure are the main risk factors for the development of DN. 30%–40% of patients with diabetes develop DN, of which 5%–10% eventually progress to end-stage renal disease (ESRD). The present study was performed for the evaluation of Angiotensin II levels and their relationship with type 1 and type 2 diabetes mellitus and diabetic nephropathy in Al-Qadisiyah Province. To achieve this aim, 100 samples were collected, divided into 2 groups: those who are 40 Type 1 diabetes patients (20 T1DM with nephropathy and 20 T1DM without nephropathy); 40 Type II diabetes patients (20 T2DM with nephropathy and 20 T2DM without nephropathy); and healthy controls. Samples were collected from Al-Diwaniyah Teaching Hospital and Diabetes Center in Al-Qadisiyah Province from March 2022 to December 2022. Five milliliters of venous blood samples were obtained from each person under sterile conditions. Serum Ang-II (Angiotensin II) concentrations were estimated by an enzyme-linked immunosorbant assay. The data were analyzed using a variety of statistical methods. The results showed a significant ($p < 0.05$) decrease in serum Ang-II (Angiotensin II) in all patient groups, compared to diabetic subjects and healthy controls. Conclusions. Ang-II (Angiotensin II) is considered a very good diagnostic marker in type 1 diabetes and a good diagnostic marker in type 2 diabetes mellitus and diabetic nephropathy. This indicates that angiotensin II plays an important role in the development and progression of diabetic nephropathy.
Keywords: Diabetic nephropathy (DN), Diabetes Mellitus, Angiotensin II.

1. Introduction

Diabetes Mellitus (DM) is one of the most prevalent chronic diseases in the world and constitutes one of the greatest public health challenges of the 21st century (Zimmet et al., 2016).
Different classes of diabetes mellitus, type 1, type 2, gestational and other types are compared in terms of diagnostic criteria, etiology and genetics. The Molecular genetics of diabetes has received extensive attention in recent years. One large set of mutations and single nucleotide polymorphisms in genes that play a role in the various steps and pathways involved in glucose metabolism and in the development, control and function of pancreatic cells have been studied (Kharroubi and Darwish, 2015).
Diabetic nephropathy is a chronic complication of both type 1 DM (beta cell destruction – absolute lack of insulin) and type 2 DM (insulin resistance and/or decreased secretion of insulin) (Vrhovac et al., 2008). Since its discovery in 1898 by Tigerstedt and Bergman, the renin system angiotensin (RAS) is widely studied. In the classical systemic signaling cascade, the juxtaglomerular cells secrete renin into the circulation, which cleaves the angiotensinogen originating mainly from the liver, into angiotensin I (Ang I). This, for in turn, it is cleaved by the Ang I converting enzyme (ACE), forming angiotensin II (Ang II). Ang II can be cleaved by peptidases (aminopeptidase - Amp or dipeptidyl aminopeptidase - D-Amp) and form respectively the Ang (III) and (IV) (Danziger, 2008 ; Wolf et al., 2000 ; wolf et al., 2002). Ang II cleavage and Ang (1-7) formation, which is detected in several tissues, including kidneys, heart, liver, spleen, lung, and central nervous system (Danziger, 2008 ; Zimmerman and Burns, 2012). The renin-angiotensin system (RAS) may be stimulated or suppressed in diabetic patients, according to literature reports (Kennefick and Anderson, 1997 ; Hollenberg et al., 2003). Hyperglycemia, angiotensin II and mechanical stress also stimulate the production the VEGF (Vascular Endothelium Growth Factor) (Leehey et al ., 2000).
VEGF is an important cytokine in the pathogenesis of diabetic nephropathy. (Chiarelli et al., 2000).

2. Experimental

The samples were taken from 100 person , divided into 2 groups: those who are 40 Type 1 diabetes patients (20 T1DM with nephropathy and 20 T1DM without nephropathy); 40 Type II diabetes patients (20 T2DM with nephropathy and 20 T2DM without nephropathy); and healthy controls. Samples were collected from Al-Diwaniyah Teaching Hospital and Diabetes Center in Al-Qadisiyah Province from March 2022 to December 2022. Five milliliters of venous blood samples were obtained from each person under sterile conditions.

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2.1 Detection of Human Ang-Ⅱ (Angiotensin Ⅱ) by Enzyme linked Immunosorbent Assay (ELISA) technique

21.1 Test Principle

This ELISA kit uses the Competitive-ELISA principle. The micro ELISA plate provided in this kit has been pre-coated with Human Ang-Ⅱ.During the reaction, Human Ang-Ⅱ in the sample or standard competes with a fixed amount of Human Ang-Ⅱ on the solid phase supporter for sites on the Biotinylated Detection Ab specific to Human Ang-Ⅱ. Excess conjugate and unbound sample or standard are washed from the plate, and Avidin conjugated to Horseradish Peroxidase (HRP) are added to each microplate well and incubated. Then a TMB substrate solution is added to each well. The enzyme- substrate reaction is terminated by the addition of stop solution and the color change is measured spectrophotometrically at a wavelength of 450 nm ± 2 nm. The concentration of Human Ang-Ⅱ in the samples is then determined by comparing the OD of the samples to the standard curve.

Kit components & Storage		
Item	Specifications	Storage
Micro ELISA Plate (Dismountable)	8 wells ×12 strips	-20℃, 6 months
Reference Standard	2 vials	
Concentrated Biotinylated Detection Ab (100×)	1 vial, 120 uL	
Concentrated HRP Conjugate (100×)	1 vial, 120 μL	-20℃(Protect from light), 6 months
Reference Standard & Sample Diluent	1 vial, 20 mL	4℃, 6 months
Biotinylated Detection Ab Diluent	1 vial, 14 mL	
HRP Conjugate Diluent	1 vial, 14 mL	
Concentrated Wash Buffer (25×)	1 vial, 30 mL	
Substrate Reagent	1 vial, 10 mL	4℃(Protect from light)
Stop Solution	1 vial, 10 mL	4℃
Plate Sealer	5 pieces	
Product Description	1 copy	
Certificate of Analysis	1 copy	

2.2 Assay procedure

1. Add the Standard working solution to the first two columns: Each concentration of the solution is added in duplicate, to one well each, side by side (50 uL for each well). Add the samples to the other wells (50 uL for each well). Immediately add 50μL of Biotinylated Detection Ab working solution to each well. Cover the plate with the sealer provided in the kit. Incubate for 45 min at 37℃. Note: solutions should be added to the bottom of the micro ELISA plate well, avoid touching the inside wall and causing foaming as much as possible.
2. Aspirate or decant the solution from each well , add 350 uL of wash buffer to each well. Soak for 1~2 min and aspirate or decant the solution from each well and pat it dry against clean absorbent paper. Repeat this wash step 3 times. Note: a microplate washer can be used in this step and other wash steps.
3. Add 100 μL of HRP Conjugate working solution to each well. Cover with the Plate sealer. Incubate for 30 min at 37°C.
4. Aspirate or decant the solution from each well, repeat the wash process for five times as conducted in step 2.
5. Add 90 μL of Substrate Reagent to each well. Cover with a new plate sealer. Incubate for about 15 min at 37°C. Protect the plate from light. Note: the reaction time can be shortened or extended according to the actual color change, but not more than 30min.
6. Add 50 μL of Stop Solution to each well. Note: adding the stop solution should be done in the same order as the substrate solution.
7. Determine the optical density (OD value) of each well at once with a micro-plate reader set to 450 nm.

2.3 Calculation of results

Average the duplicate readings for each standard and samples. Plot a four-parameter logistic curve on log-log graph paper, with standard concentration on the x-axis and OD values on the y-axis.

If the samples have been diluted, the concentration calculated from the standard curve must be multiplied by the dilution factor. If the OD of the sample is under the lowest limit of the standard curve, you should re-test it with an appropriate dilution. The actual concentration is the calculated concentration multiplied by the dilution factor.

3. Results

3.1 Ang- II levels in T1DM patients and control groups.

To evaluate the Ang-II cutoff value as well as to predict the T1DM as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (1), and figure (1). The Ang-II cutoff value was < 182.91 with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Area under curve of 82.5 %, 80.0 %, 89.2 %, 69.6% and 0.865 (0.754- 0.975). The present results indicates Ang-II is considered as a very good diagnostic marker.

Table (1): Sensitivity and specificity of Ang-II level (< 182.91-fold) in T1DM patients		
Ang-II level (fold)	T1DM n = 40	Control n = 20
< 182.91	33 (%)	4 (%)
> 182.91	7 (%)	16 (%)
Sensitivity %	82.5 %	
Specificity %	80.0 %	
PPV %	89.2%	
NPV %	69.6 %	
AUC (95% CI)	0.865 (0.754- 0.975)	
CI: Confidence interval, AUC: Area under curve, NPV: Negative predictive value, PPV: Positive predictive value.		

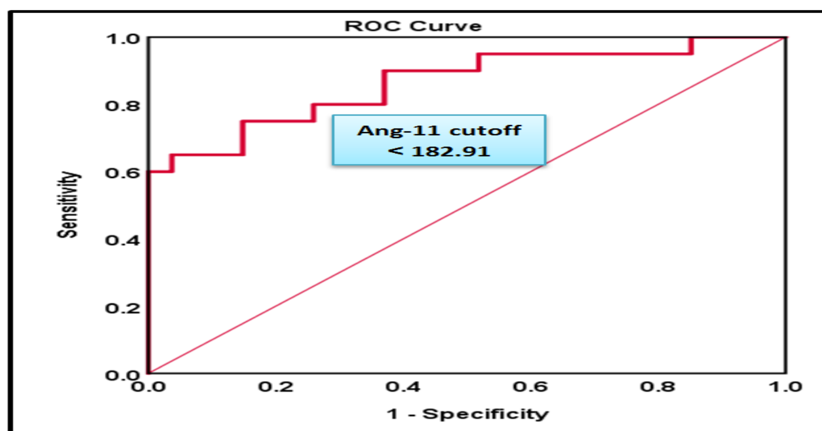

Figure (1): Receiver operator characteristic curve analysis for the calculation Ang-II possible diagnostic cutoff value of T1DM.

3.2 Ang-II levels in T2DM patients and control groups

To evaluate the Ang-II cutoff value as well as to predict the T2DM as diagnostic tests or adjuvant diagnostic tests, receiver operator characteristic (ROC) curve analysis was carried out and the results are shown in table (2), and figure (2). The Ang-II cutoff value was < 307.94 with sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and Area under curve of 77.5 %, 70.0 %, 83.3 %, 60.9% and 0.777 (0.635- 0.918). The present results indicates Ang- II is considered as a good diagnostic marker.

Table (2): Sensitivity and specificity of Ang-II level (< 307.94-fold) in T2DM patients		
Ang-II level (fold)	T2DM n = 40	Control n = 20
< 307.94	31 (%)	6 (%)
> 307.94	9 (%)	14 (%)
Sensitivity %	77.5 %	
Specificity %	70.0 %	
PPV %	83.3%	
NPV %	60.9 %	
AUC (95% CI)	0.777 (0.635- 0.918)	
CI: Confidence interval, AUC: Area under curve, NPV: Negative predictive value, PPV: Positive predictive value.		

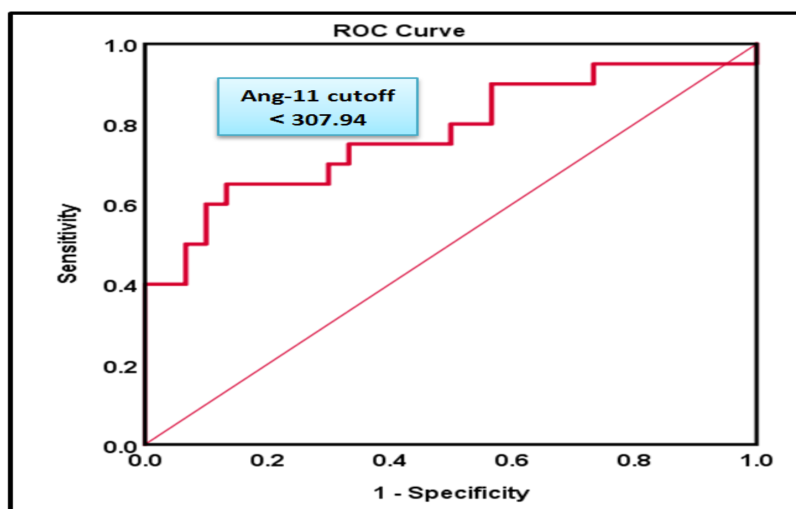

Figure (2): Receiver operator characteristic curve analysis for the calculation Ang-II possible diagnostic cutoff value of T2DM.

3.3 Ang- II level in Patients and healthy controls

Serum levels of Ang-II were measured for all participants and results are shown in table 3. Mean levels of Ang-II were 256.1 ± 12.7 pg/ml, 130.7± 7.22 pg/ml, 261.6± 6.89 pg/ml, 288.3 pg/ml and 1166.4± 35.7 pg/ml, in T1DM with nephropathy, T1DM without nephropathy, T2DM with nephropathy, T2DM without nephropathy and healthy control group respectively; the mean levels was significantly decrease in all patient groups in comparison with control groups (P= 0.001).

Table (3): Ang-II level in in Patients and healthy controls.					
	Cases–control comparison				
	T1DM With DN n=20	*T1DM Without DN n=20*	*T2DM With DN* n=20	*T2DM Without DN n=20*	*Control n=20*
Ang-II Pg/ml					
Mean± SD	256.1±12.7^{A}	130.7± 7.22^{A}	261.6±6.89^{A}	288.3± 8.1^{A}	1166.4±35.7^{B}
P. Value	0.001 S				
Different latters denote to the significant differences at p< 0.05 SD: standard deviation; Test: one way ANOVA; S: significant at P < 0.05.					

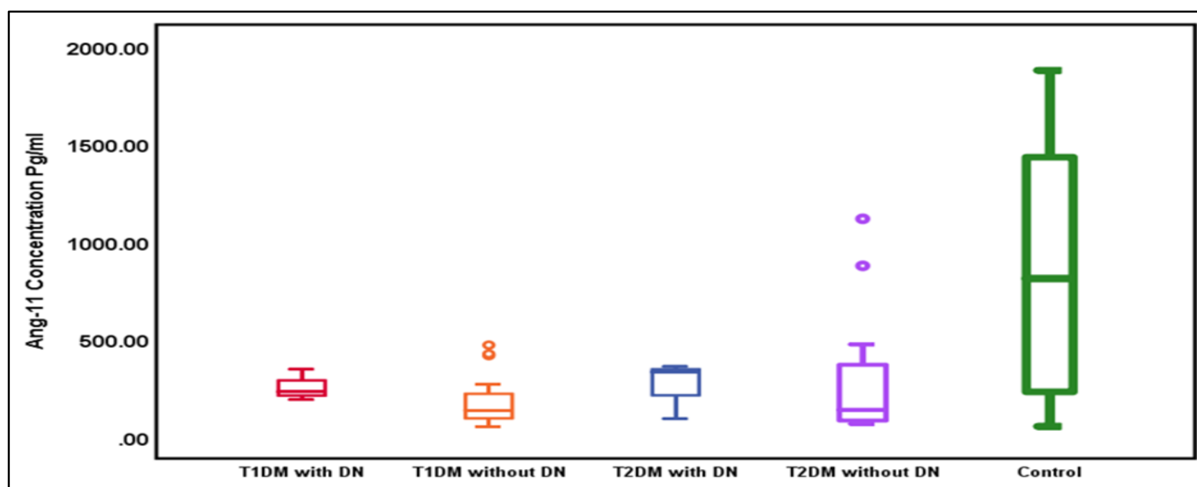

Figure (3): Box plot showing comparison of median serum Ang-II level among patients and control subjects.

33.1 Frequency distribution of Ang-II levels according to gender of patients and healthy controls

The comparison of Ang-II levels according to gender of patients and healthy controls has been carried out and the results were demonstrated in table (4). The mean levels of Ang-II were non-significantly higher in female groups in comparison of male groups in all study groups.

Table (4): Frequency distribution of Ang-II levels according to gender of patients and healthy controls			
Groups	Male–Female comparison		
	Male	*Female*	*P value*
Ang-11			
T1DM	182.8 ± 15.01^{A}	185.48 ± 20.83^{A}	0.955
T2DM	284.85± 42.6^{A}	286.19 ±51.15^{A}	0.991
Control	1010.65 ±50.72^{B}	1192.99 ±10 4.38^{B}	0.592
P. Value	0.001	0.001	
In each Colum: Different latters denote to the significant differences at p< 0.05			
Test: one way ANOVA; S: significant at P ≤ 0.05. NS: non-significant at P > 0.05.			

4. Discussion

Our current results are not consistent with the study that found that plasma angiotensin II levels were increased in non-insulin dependent diabetic patients with nephropathy (Nicola et al., 2001). However, another study found that during the course of diabetes, plasma renin activity and angiotensin II levels are normal or low in some diabetic patients (Wolf and Ziyadeh, 1997).

Additionally, a review article suggests that angiotensin II shows increased activity during diabetic nephropathy, which causes hypertrophy of various renal cells (Chawla et al., 2010).

Moreover the present results is noncongruent with the findings of a study conducted by Yousef et al. they reported that In diabetic patients with nephropathy there is higher ACE activity compared with diabetic patients without nephropathy. The increased protein expression of ACE is responsible for high level of angiotensin II. Angiotensin II increases the podocyte injury and loss of podocytes is a hallmark of progressive kidney diseases including

DN. In addition to its hemodynamic effects in diabetic kidney as it increases the intraglomerular pressure and glomerular filtration rate . Angiotensin II stimulates the release of several cytokine mediators of glomerulosclerosis, such as osteopontin, platelet-derived growth factors, fibonectin, and transforming growth factors B, finally leading to ESRD (Yousef et al., 2014), and the higher rate of Ang- II among in T2DM with no DN is likely due to the fact that these patients are older, less healthier at diagnosis, and have a lot co-morbidities and the mixed etiology than patients with T1DM (Hoogeveen, 2022).

In addition, the current study showed that mean levels of Ang-II were non-significantly higher in female groups in comparison of male groups in all study groups.

This result inconsistence with the result done by Ribeiro-Oliveira et al. they reported that Ang-11 in diabetics have shown conflictive results regarding the activation of this system. For example, in diabetes nephropathy, studies have shown a suppression of Ang-11 in plasma level (Ribeiro-Oliveira et al., 2008).

As shown in this study by Nicola et al. they concluded that Plasma angiotensin II is decreased in normo albuminuric diabetics and elevated in diabetic nephropathy regardless the sex of the patient (Nicola et al., 2001).

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