

# Assessment of serum Cystatin-C, Cortisol and Aldosterone hormone as markers for early detection of nephropathy among Iraqi patients with Type 2 Diabetes Mellitus

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**ABSTRACT:** Biomarkers which identify Diabetic nephropathy (DN) patients could allow early, more efficient intervention and management, decreasing patient morbidity and mortality. This study aimed to estimate the predictive value of cystatin-c protein, and the hormones cortisol and aldosterone in detecting DN in a study including 60 patients with DN under hemodialysis (HD) and other 60 patients with type 2 diabetes mellitus (T2DM) who were recruited from AL-Yarmouk, AL-Kindy and AL- Karama Teaching Hospitals' dialysis center. Serum levels of cystatin-c, cortisol, and aldosterone were measured for both groups. Serum cystatin-c protein, cortisol and aldosterone hormones levels were significantly higher in patients with DN under HD when it compared with T2DM patients. Receiver operating characteristic (ROC) curve was utilized to test the cut off values of these biomarkers to predict DN results revealed that serum cystatin-c, cortisol and aldosterone levels were significantly higher in patients with DN under HD when it compared with T2DM patients. ROC analysis revealed that the cystatin-C level had the best predictive value in detecting of DN compared with serum cortisol and aldosterone which indicate that cystatin-C could be used to predict DN development and progression in patients with T2DM.

**KEYWORDS:** Diabetes mellitus; Diabetic nephropathy; Cystatin-c; Cortisol; Aldosterone.

## 1. INTRODUCTION

Diabetes mellitus (DM) is considered as one of the most challenging health issues in the current era. Type2-diabetes mellitus (T2DM) represent about (85% - 95%) of total diabetic issues in developed nations and countries and accounts for biggest percentage in leading countries [1]. Individuals diagnosed with T2DM are inherently susceptible to tissue damage in organs that are supplied by an end arterial system as a result of microangiopathy. These microvascular complications Include diabetic nephropathy (DN), diabetic retinopathy, and diabetic neuropathy. The progression of DN begins with the emergence of abnormally low amounts of albumin in urine ( $\geq 30$ mg - 299 mg/day), known as microalbuminuria. This then advances to the stage of macro albuminuria ( $\geq 300$ mg/day), and ultimately leads to End-Stage Renal Disease (ESRD). Micro-albuminuria may still under progression to macro-albuminuria in (10-15 years) [2,3]. Epidemiological researches have clarified that the mortality ratio of DM patients with DN is 30 time higher than the patients without kidney disease complications; so, earlier characterization and medication of DN is essential for developing the quality of life (QOL) of DM patients [4].

Cystatin-C (Cys-C), is a non-glycosylated with low molecular weight protein (13.4 k Da) belonging to the cystatin superfamily of cysteine protease inhibitors [5]. It's produced at a constant rate by all nucleated cells and is expressed in relatively high concentrations in serum, saliva, seminal, and cerebrospinal fluids [6]. Unlike creatinine, it does not undergo renal secretion from tubule and is not influenced by extra-kidney factors like age, race, gender, diet, and muscle mass, so Cys-C levels are a more sensitive indicator of kidney function and estimate the GFR than serum creatinine levels [7,8].

Cortisol (hydrocortisone), a hormone belongs to the glucocorticoid hormones, is produced by the adrenal glands. It assists cells to make energy [9]. When a body experiences stress, even it was physical,

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mental, or emotional, cortisol reproduction by the adrenals is increased in order to conserve the body. Patients with T2DM and chronic kidney disease (CKD) Individuals encounter numerous stressful circumstances in their daily lives. The majority of them exhibit several co-morbidities and have diverse social, economic, and medical challenges. Since T2DM and chronic kidney disease (CKD) patients are living in stressful conditions; it may be feasible that cortisol concentrations could be connected with these circumstances [10].

The renin-angiotensin-aldosterone system (RAAS) is essential causes in the pathogenesis of DN. The activation of the local RAAS leads to a rise in angiotensin II, as well as the activity of adrenocorticotrophic hormone (ACTH) and potassium. These three variables are primarily responsible for regulating aldosterone secretion [4]. Aldosterone stimulates the reabsorption of sodium through the mineralocorticoid receptor on cells lining the renal tubules. This leads to an increase in fluid volume and blood pressure, which is strongly associated with proteinuria and the progression of kidney injury [11,12]. The purpose of the present study is to evaluate the diagnostic value of Cys-c protein and hormones cortisol and aldosterone for discriminating between T2DM and DN.

## 2. RESULTS

### 2.1. Demographic baseline of the study population

As indicated in Table 1, no significantly differences were obtained in age among patients in DN under hemodialysis (HD) and T2DM patients. Body mass index (BMI) was significantly higher in T2DM patients (32.95±3.08) compared with DN under HD patients (27.18±5.58). The duration of DM in patients with DN under HD was significantly higher than patients with T2DM without DN (7.0years vs. 3.5 years).

Table 1. Demographic profile of the study sample

Variable	DN under HD (n=60)	T2DM (n=60)	p-value
<b>Age, years</b>			
Mean ±SD	46.37±12.07	44.6±5.41	0.762
Range	30-72	30-70	
<b>BMI, Kg/m<sup>2</sup></b>			
Mean ±SD	27.18±5.58	32.95±3.08	<0.001
Range	16.16-46.81	24.72-36.73	
<b>Duration of DM, M</b>			
Mean ±SD	114±3.85	4.11±3.12	<0.001
Median	7.0	3.5	
Range	1.0-15.0	0.1-12.0	

### 2.2. Determination of biochemical tests

Table 2 demonstrated that mean value of HbA1c in Patients with T2DM (8.31±1.34%) was higher than patients with DN under HD (6.6±1.73%). In the same table, the results revealed that there were non-significant difference in levels of FBG between patients with DN under HD and T2DM patients (181.32±44.2 mg/dl and 181.68±39.41 mg/dl, respectively). The mean value of blood urea and creatinine in the patients with DN under HD were (153.83±47.04 mg/dl and 8.08±2.54 mg/dl, respectively) compared 35.80±7.55 mg/dl and 1.48±2.10 mg/dl, respectively in T2DM with highly significant differences. While, GFR was significantly lower in patients DN under HD (7.6±2.90 ml/min/1.73m<sup>2</sup>) than those with T2DM (96.12±12.30 ml/min/1.73m<sup>2</sup>).

Table 2. Mean ±SD values of biochemical tests

Variable	DN under HD (n=60)	T2DM (n=60)	p-value
<b>FBS, mg/dl</b>			
Mean ±SD	181.32±44.2	181.68±39.41	0.962
Range	133-320	130-248	
<b>HbA1c, %</b>			
Mean ±SD	6.6±1.73	8.31±1.34	<0.001
Range	4.3-11.5	6.3-11.3	
<b>Urea, mg/dl</b>			
Mean ±SD	153.83±47.04	35.80±7.55	<0.001
Range	28.7-270.41	20.0-64.0	
<b>Creatinine, mg/dl</b>			
Mean ±SD	8.08±2.54	1.48±2.10	<0.001

<b>Range</b>	4.04-13.48	0.50-9.0	
<b>GFR, ml/min/1.73m<sup>2</sup></b>			
<b>Mean ±SD</b>	7.6±2.90	96.12±12.30	<b>&lt;0.001</b>
<b>Range</b>	4.0-14.0	64.0-121.0	

### 2.3. Determination of Cystatin-C, Cortisol and Aldosterone

As depicted in table 3, the results showed that the median value of the Cys-C was significantly higher in DN under HD patients (25.75 ng/ml) compare with T2DM patients (8.3 ng/ml). The median serum level of cortisol in patients with T2DM was significantly lower than that of patients with DN under HD (median= 10.51 pg/ml, 14.43 pg/ml, respectively). Also, aldosterone level was significantly higher in patients with DN under HD (median= 56.73 ng/ml, range= 17.81-179.4 ng/ml) than patients with T2DM (median= 24.48 ng/ml, 11.09-59.53 ng/ml).

**Table 3.** Cys-C and hormonal profile among study population.

Variable	DN under HD(n=60)	T2DM (n=60)	p-value
<b>Cys-c, ng/ml</b>			<b>&lt;0.001</b>
<b>Mean±SD</b>	30±34.71	8.76±1.34	
<b>Median</b>	25.75	8.3	
<b>Range</b>	10.0-292	6.50-12.2	
<b>Cortisol, mcg/dl</b>			<b>0.008</b>
<b>Mean±SD</b>	15.14±7.35	11.88±5.75	
<b>Median</b>	14.43	10.51	
<b>Range</b>	2.15-40.86	3.36-38.19	
<b>Aldosterone, pg/ml</b>			<b>&lt;0.001</b>
<b>Mean±SD</b>	58.47±29.16	26.51±11.46	
<b>Median</b>	56.73	24.48	
<b>Range</b>	17.81-179.4	11.09-59.53	

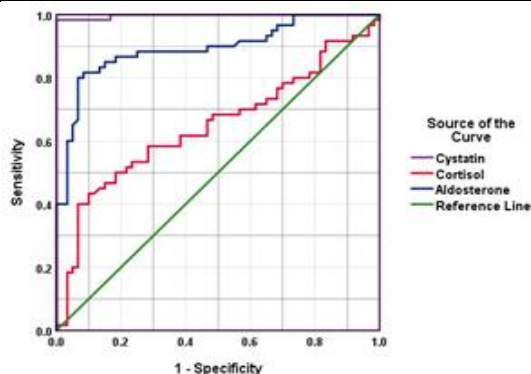
### 2.4. ROC analysis of cystatin-c, cortisol and aldosterone

The Receiver operating characteristic (ROC) curve analyses of cystatin-c, cortisol and aldosterone in anticipating DN in patients with T2DM are depicted in Figure 1 and Table 4.

The area under the curve to diagnose established diabetic nephropathy in type 2 DM using Cys-C was 0.997, 95%CI=0.991-1.0, p< 0.001 with specificity and sensitivity 100% and 98%, respectively at cut-off value of = 14.65 ng/ml. For cortisol, the AUC was 0.647, 95%CI= 0.547-0.748, p= 0.001. The sensitivity and specificity at cut off value of cortisol = 12.75 mcg/ml were 62% and 61%, respectively. For aldosterone, the AUC was 0.893, 95%CI=0.833-0.953, p< 0.001. The sensitivity and specificity at cut off value of aldosterone = 34.34 pg/ml were 87% and 88%, respectively.

**Table 4.** ROC analysis results for Cys-C, Cortisol and aldosterone

Marker	AUC	95%CI	Specificity	Sensitivity	Cut-Off value	P-Value
<b>Cys-C</b>	0.997	0.991-1.0	100	98	14.65	< 0.001
<b>Cortisol</b>	0.647	0.547-0.748	61	62	12.75	0.001
<b>Aldosterone</b>	0.893	0.833-0.953	88	87	34.34	p< 0.001



**Figure 1.** ROC for cystatin-c, cortisol and aldosterone in the context of discrimination between T2DM and DN.

## 3. DISCUSSION

Many individuals with T2DM undergoes a phase of pre-diabetes and may encounter renal impairment. Indeed (20-40%) of people with diabetic will Advancement to more grave renal disorders e.g.

DN. So, detection of renal dysfunction at earlier stage is substantial as early Interference can slow down the loss of kidney role, that Enhances survival and life quality [2,13]. It is obvious from the results of the current study that the mean age of DN patients under HD was not- significantly differ from that of non-DN T2DM patients. These results are compatible with previous studies which reported that a non-significant difference in age was observed between DN patients under HD and non-DN T2DM [14, 15]. It has been suggested that the prevalence of CKD is increasing in the elderly can be due to an increase in age related risk factors for progression to the CKD such as diabetes, hypertension and cardiovascular disease [16]. Furthermore, BMI were significantly higher in non-DN T2DM patients compared with DN patients under hemodialysis in agreement with results reported previously [17, 18]. Body mass index is widely utilized as a risk factor in the development or prevalence of several health problems. The present results reported previously which stated that individuals with BMI  $\geq 30$  kg/m<sup>2</sup> are at substantially heightened lifetime risk of diabetes, having even moderately elevated BMI is associated with increased risk of developing diabetes mellitus complications [19]. The BMI was lower in patients with hemodialysis as this condition represents a chronic depletion condition that affects or increases metabolic rate and causes severe weight lost in addition to frequent dialysis and strict dietary habits that reduces protein intake [20].

Results obtained demonstrated that mean FBG did not differ significantly between patients DN under HD and non-DN T2DM patients .Whereas HbA1c in patients with non-DN T2DM patients was higher significantly than that of DN patients under HD in agreement with results demonstrated previously [14, 17, 18].

The explanation of these findings might be due to that during hemodialysis, the uremic environment, blood loss during treatments, and frequent phlebotomy all contribute to decreased RBC lifespan. So shortened RBC survival and red cell transfusions are likely to lower the HbA1c. Also, lower HbA1c values were associated with lower hemoglobin concentration and higher doses of erythropoietin [3, 4]. The glycation rate of just-produced young erythrocytes is reported to be lower than that of old cells. Therefore, it seems that the decrease of HbA1c levels relative to FBG in HD with diabetes patients and are treated with erythropoietin might be due to the increasing proportion of young erythrocytes over old erythrocytes in peripheral blood of those patients [21, 22].

Serial determinations of serum Cys-C accurately diagnosed trends in kidney function in people with normal or high glomerular filtration rate (GFR) and offer a method for investigating early loss in renal function in individuals with diabetes [23]. The findings of our study indicated that a significant proportion of individuals with diabetes experienced an elevation in their serum levels of Cys-C. Consistent with previous research, our findings indicate that Cys-C may have a function in the progression of DN in individuals with diabetes. [4]. Many previous studies have provided evidences that serum Cys-C is a better sensitive biomarker for diagnosis of earlier changes for in glomerular filtration in T2DM patients than and creatinine, because of its independence of age, BMI and gender. Also, other study was Performed by Gupta et al. clarify that the Cys-C concentrations were elevated even in the patients that clinical albuminuria were not started yet, Therefore, it serves as an earlier indicator than microalbuminuria in identifying nephropathy [24,25]. The limited capacity of creatinine to identify early deterioration in glomerular filtration rate (GFR) is attributed to the fact that serum creatinine level (SCr) only starts to elevate above the normal value when approximately 50% of renal function is losted, suggesting that GFR can change before SCr going to be abnormal [13].

Studies that conducted on rats observe that renal clearance of Cys-C is 94% of the Often-utilized GFR parameters Cr EDTA and that Cys-C thus is actually filtered in the glomeruli freely. Under normal circumstances, the filter of glomerular is clears compounds with MWT up to 58,000 Dalton while in ESRD these solutes are retained i.e. Cys-C leading to its increased serum concentrations [26]. This explains the high level of Cys-C in DN under HD compared with T2DM in the present study.

Recently, Sun, S. and Wang, Y. investigated whether the levels of blood cortisol are linked to the occurrence of microvascular complications in patients with (T2DM). The level of cortisol had a favorable correlation with the degree of microalbuminuria, as well as with the severity of diabetic retinopathy and neuropathy [27]. The authors speculated that raised excretion of cortisol may be contributed to the advancement of diabetes and its emergence of micro-albuminuria. Zhang, X., et al ,2020 evaluate the relationship among serum cortisol concentrations and the existence of micro albuminuria in patients with T2DM and pre-diabetes. Serum levels of cortisol were significantly higher in microabuminuria group than in normoalbuminuria group. After amendment for multiple factors, the correlation among cortisol concentration and microalbuminuria remained consistent and significant. The authors concluded that

increased values of cortisol, even when it is in the normal value, may be connected to the Progress of micro albuminuria [28]. The explanation for such an association was that the high level of cortisol may lead to the raise of visceral fat amount, that in turn leads to the raise of plasma free fatty acid (FFA) values. An excess amount of FFA would attach to serum albumin, leading to an increase in the excretion of albumin into the urine. Elevated levels of FFA can potentially trigger the activation of the protein kinase-C (PKC) pathway in kidney vascular endothelial cells, that has been demonstrated to have a significant impact on the development of DN [29].

Aldosterone is a key risk factor promoting inflammation and fibrosis leading to renal complications. Plasma aldosterone concentration is elevated in patients with diabetes mellitus [30]. Denecke B., et al, 2003 Exhibited that the plasma aldosterone value is inversely related with insulin sensitivity [31]. Furthermore, there is enormous experimental realities that aldosterone contributes to the Progress of nephron-sclerosis and kidney fibrosis in exemplars of diabetes and hypertension [23].

Previous data showed that elevated levels of circulating aldosterone are associated with a malfunction in the insulin-signaling pathway and impaired functioning of the endothelium, resulting in insulin resistance and injury to the kidneys [33]. Various mechanisms have been suggested to explain the development of insulin resistance caused by aldosterone, including altered function of pancreatic  $\beta$ -cells, reduced insulin sensitivity in target organs, and the promotion of deleterious adipocytokine production in adipose tissue [34].

Finally, the present study found as shown in figure 1 that Cys-C had sensitivity and specificity higher than cortisol and aldosterone hormone; therefore, Cys-C could be a good and an earlier marker for the diagnosis of nephropathy in patients with T2DM.

#### 4. CONCLUSION

The results obtained from this research work supposed that the evaluation of Cys-C protein in serum of patients having T2DM is a more efficient method than cortisol and aldosterone hormones for early detection of kidney dysfunction in patients with T2DM. These findings need further investigation to elucidate the exact role of Cys-C protein as a diagnostic marker for DN.

#### 5. MATERIALS AND METHODS

##### 5.1. The Study Population

This study included 120 patients with T2DM, ages range 30-72 years. They were recruited from AL-Karama Teaching Hospital, AL-Yarmouk Teaching Hospital, and AL-Kindy Teaching Hospital in Baghdad / Iraq for the period from January 2022 to June 2022. The participants were divided into two primary categories: 60 patients without DN and 60 patients with DN undergoing hemodialysis treatment 3 times a week for 3 hours/ session.

Exclusion criteria included the individuals with Type I diabetes mellitus, hypo or hyperthyroidism, clinically confirmed depression, and has undergone renal transplantation, other patients with renal disease such as systemic lupus erythematosus. A comprehensive questionnaire comprising specific demographic information such as gender, age, and duration of diabetes, hemodialysis (HD) duration, each participant's treatment type (whether dietary, oral, insulin, or combined therapy) and parents' history of diabetes were collected. Anthropometric measurements, including height and body weight, and body mass index (BMI) were evaluated in all patients subjected to the study according to standard protocols.

##### 5.2. Sample collection

About 10 mL of venous blood were drawn from patients with T2DM and DN under HD (before taking the dose of heparin) by venipuncture, and splitted to two aliquots; 5 ml in ethylene diamine tetra acetic acid (EDTA) (1.5 mg/mL) for determination of HbA1c%, while the second aliquot were moved into plain tube without anticoagulant, permitted to clump for thirty minutes, centrifuged for 15 minutes at 3000 xg. Separated sera were frozen in tightly sealed Eppendorf tubes and stored at -20°C for biochemical tests.

##### 5.3. Biochemical Tests

Ready commercial kits (Elecsys / Roche Diagnostics GmbH, Germany) were used for measuring serum level of cortisol hormone. The cobas e 411 instrument is a full automated analyzer which utilized a patented Electro ChemiLuminescence (ECL) technology of immunoassay analysis for cortisol hormone. A ready commercial kit (Melsin/China) was utilized to quantify serum concentration of Cys-C and aldosterone

hormone using enzyme linked immune-sorbent assay. Absorbance was taken at 450 nm and a standard curve was calculated from the standard dilution of Cys-C and aldosterone hormone.

#### 5.4. Statistical analysis

The results of the present study have been analyzed by using SPSS version 25.0 (SPSS, Chicago). Continuous results were presented as mean and SD (or median and range as required), and analyzed by the use of Student t-test. ROC analysis was utilized to estimate the predictive level of cystatin-C, cortisol and aldosterone hormone in predicting DN among patients with T2DM. A p-value of <0.05 was regarded to be significant [35,36].

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