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Investigate some Biochemical Parameters and Impact of NOS3 Gene Polymorphism among Iraqi Patients with Chronic Kidney Disease

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Abstract: Chronic kidney disease CKD as a kidney damage with persistence about three months or longer, as well as it represents an especially large load in low- and middle-income countries, which are less equipped to deal with its consequences. Chronic kidney disease has revealed as one of the leading causes of mortality worldwide. The aim of the current study was to investigate the association between genetic polymorphism of NOS3 genes and the severity of CKD in Iraqi population. Fifty Iraqi CKD patients (males 17 and females 33) and fifty apparently healthy as control group (male27 and females23) aged 20 –65years old were involved in the study. They were diagnosed by specialist physicians and estimation GFR<15 were chosen as CKD patients. The result of the current study according to the demographical distribution gave different outcomes, in relation to age were significant the highest percentage (34%)of the disease appeared and it was diagnosed at age 50-59 years old. The sex result show no significant differences between the two studied groups. According to the clinical aspect of diagnosis of patient were depended on several criterial basically it is measuring the level of serum creatinine and urea blood the result was significant (P≤0.05) in CKD patients 12.43±6.20and 136.26±38.80 respectively, *Versus* the control group 0.64±0.11, 20.58±4.55. The results showed a significant decrease in both hemoglobin and iron levels in CKD patients 9.00±1.84, 10.17±7.24 respectively, *Versus* the control group 13.12±1.75, 18.80±3.97. More over according to ferritin shows a significant increase in the serum level for CKD patients 506.54±86.16 *Versus* the control group 126.18±72.56 and according to hypertension, whereas 92% of the CKD patients are hypertension,with respect to NOS3 gene polymorphisms, allele and genotype frequencies showed significant differences between CKD patients and apparently healthy individuals. The Nos3 gene located in intron on chromosome 7, it involves a polymorphism (C to T, rs 2070744). The study found that carriers with the TT and CT genotype showed statistically significant differences at a significant level of p≤0.05 and OR more than 1. As a conclusion of the current study the effect of the variant allele for SNPs (rs 2070744C>T) of NOS3 gene appears to be related with chronic kidney disease, that suggests to have a modifier effect on the disease incidence.

Keywords: *NOS3* gene polymorphisms (*rs2070744*), CKD Iraqi patients, Chronic Kidney disease.

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Introduction

Chronic kidney disease (CKD) as kidney damage with persist about three months or longer. As well as it represents an especially large load in low- and middle-income countries, which are least equipped to deal with its consequences. Chronic kidney disease

has reveal as one of the leading causes of mortality worldwide, and it is one of a small number of non-communicable diseases that have shown an increase in associated deaths over the past 2 decades (1). Damage can present with structural or functional kidney alterations, with or without decreased glomerular filtration. The structural modifications can be evidenced histologically, radiologically or by biochemical markers of kidney damage in serum or urine samples (2), moreover is a complex disease which affects approximately 13% of the world's population. Additionally, can cause renal dysfunction and progression to end-stage kidney disease and cardiovascular disease. Complications associated with disease may contribute to the disease progression and the risk of cardiovascular-related morbidities, as early CKD is asymptomatic, and symptoms only present at later stages when complications of the disease arise, such as a decline in kidney function and the presence of other medical conditions fellow with the disease (3). The prevalence of CKD has been reported to be higher in females than in males. CKD affects populations in different regions of the world unequally, likely as a result of differences in population demographic characteristics, their comorbidities, and access to health care resources (3). The results of a genome-wide association study showed that several loci were associated with CKD and estimated glomerular filtration rate (eGFR) (4) as well, CKD displays familial clustering that may be explained by the dual effects of genetic susceptibility and environmental exposure (5). One of the factors that regulate vascular tone and influence endothelial dysfunction is nitric oxide. This compound is synthesized in the vascular endothelium by the action of the enzyme nitric oxide synthase (NOS) NOS3, which has 23.605 bases and is located at 7q36.1. It has 26 exons and encodes for eNOS, an enzyme composed of 1203 amino acids with a molecular weight of 133 289 Da. Renal nitric oxide plays several hemodynamic functions in the renal glomeruli. However, its most important effect is the promotion of diuresis and natriuresis, as well as renin secretion regulation. The eNOS is expressed in large amounts in the renal vascular endothelium (including the afferent and efferent arterioles). It is also expressed in the proximal tubule, the thick portion of the ascending loop of Henle, and the collecting tubule. -786 C > T (rs2070744). This polymorphism, situated in the flanking region 5', has been associated with a decrease in the expression of the NOS3 gene, as it decreases the transcription rate of the gene by 50%. It is thought that it can bind to the replication protein A1; this protein participates in several cellular processes, among them transcription. In studies conducted in African, Caucasian, and African-American populations, the frequency of this polymorphism is 3.2%, 14.5%, and 1.8%, respectively (2). other disease related with CKD Patients have some degree of sensorineural hearing loss and should be evaluated and evaluated to stop progression (6). According to our knowledge there was no studies about the NOS3 gene and it's correlation with CKD in Iraqi patients. This study aims to clarify the correlation between NOS3 gene polymorphisms in patients with chronic kidney disease in Iraq. But there's a study Altaie, ALSaadi and Al Saedi (7) about the ENOS gene and it's

correlation with ADPKD in Iraqi patients. While Mustafa and Abdulwahid (8) who study the correlation of *ENOS* gene with hypertension. Other study about gene ENOS wsa the expression of endothelial nitric oxide synthase (eNOS) gene represents one of the most important causes that effect the male infertility(9).

Materials and methods

Samples

The study was conducted from May 2022 to the end of September 2023. In kidney transplantation center/medical city/lmamain Al Kadhemain Medical Teaching Hospital Al-Numan Hospital, also in some of Baghdad's laboratories. The patients group include 50 CKD subjects aged (20- 65) years old diagnosed by specialist doctors, as well as control group included 50 subjects whose age matched the patient group they were Apparently healthy.Renal function tests in serum urea, serum creatinine, serum iron, serum ferritin and Hb were measured using manual perusal of patients' case sheets was used to review patient medical records. A data collection checklist was used to collect data from electronic medical records on epidemiological, clinical, laboratory, and outcome measures. In addition, patient questioner, such as symptoms, and indicators, as well as laboratory tests, such as serum creatinine, Serum urea, serum ferritin, serum iron and HB were collected for each case study (patient and control). Also two ml of blood collected from both groups and collected in the K3-EDTA thus, transfer for stored at -20 C° deep freezing and using in DNA extraction.

Genomic DNA extraction and genotyping using HRM -RT PCR technique for NOS3 polymorphism

Genomic DNA was extracted using Genomic DNA Extraction Kit (EasyPure® / Chinese) and the extraction was carried out following the manufacture instruction. HRM-RT PCR reaction was performed using QIAGEN Rotor geneQ (Germany) by applying a specific primers showed in Table (1), and the following reaction parameters. Enzyme activation 95°C for 5 min, followed by 40 cycles of amplification including denaturation at 58°C, annealing at 60°C for rs*2070744*, extension at 72°C (each comprising 15 s), Extension at 72°C for 15s and HRM at 65-95°C (0.2s for 1degree). The DNA was checked by electrophoresis on 2% agarose gels with ethidium bromide (10 ng/100 mL of agarose solution in Tris borate EDTA buffer).

Statistical analysis

Blood urea, serum creatinine, hemoglobin, iron, ferritin and Gene polymorphism data was given as mean ± standard error (SEM). Significant differences between two independent means using student t-test at 0.05 level followed by Least Significant Difference (LSD) test. Allele frequencies of NOS3gene were estimated by the direct gene counting method. Genotypes of NOS3 rs*2070744*, SNPs were given as percentage frequencies and significant differences between their distributions in CKD patients and controls were assessed by person's chia squire probability (p). In addition, odds ratio (OR) was also estimated to define the association between a genotype and CKD patients. These estimations were calculated by using the available statistical package of IBM SPSS-29.

Table (1): Primers sequences used designed in current study.

Nos3 Gene Polymorphism(rs2070744)				
SNP	Primer	Sequence (5′→3′ direction)	Product size (bp)	Tm°C
rs2070744				
C >T				
	Forward	ACCAGGGCATCAAGCTCTTC	68	60
	Reverse	CGCAGGTCAGCAGAGAGACT	68	60

Results and discussion

Demographical distribution of the study

Demographical Distribution of CKD patients according to age, sex, smoking and hypertension

According to age results of the present study revealed that there were significant differences between apparently healthy control and CKD patients. Moreover, result revealed marked differences in the percentage of patients among different ages. Whereas the highest percentage of patients was 34% at age 50-59 years and the lowest percentage 4% at age 20-29 years old. With mean age 50.2±11.9 for patients and for 44.9±12.1 apparently healthy individuals. Table (2) represent the groups of age and their significance.

Furthermore showed the results of distribution of apparently healthy controls and CKD patients according to sex. Results revealed that there were no significant influence to the sex on the incidence of the disease. Smoking is considered one of the risk factors that threating human health. Results in this study showed that smoking is one of the unrelated factors to the disease since 72% of the patients are non-smokers than apparently healthy controls was 92% Table (2). Smoking was associated with significantly higher risk of worsening kidney function (10), main potential effects of smoking on kidney is due to nicotine inflammatory and redox disorder causing endothelia damage(11, 12).

Table (2): Distribution of CKD patients and control group according to age, sex, smoking and hypertension.

		CKD patients		Controls		P value
		No.	%	No.	%	
Age (years)	>20---	2	4.0	5	10.0	0.166
	50---	17	34.0	14	28.0	
	=>60years	15	30.0	7	14.0	
	Mean±SD (Range)	50.2±11.9 (23-62)		44.9±12.1 (23-62)		0.029#
Sex	Male	19	38.0	28	56.0	0.071
	Female	31	62.0	22	44.0	
Smoking	Yes	14	28.0	4	8.0	0.009*
	No	36	72.0	46	92.0	
Hypertension	Yes	46	92.0	4	8.0	0.0001*
	No	4	8.0	46	92.0	
-Odds Ratio & its 95% Confidence interval						
*Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.						

High blood pressure is defined as a systolic blood pressure of 140 mm Hg or more, or a diastolic blood pressure of 90 mm Hg or mor (13). Hypertension is an important cause of chronic kidney disease (CKD). current result revealed

that there is significant differences between chronic kidney disease patients than apparently healthy individual according to hypertension, whereas 92% of the patients are hypertension and 8% of the healthy individual able (2). Blood pressure(BP) It is one of the leading causes of chronic kidney disease (CKD). Olsen *et al.,* (14) found BP rises with decline in glomerular filtration rate. Other researchers indicated that hypertension correlates with renal structural abnormalities (15). High BP can be either a cause or a consequence of CKD. High BP may develop early in the course of CKD and can be associated with adverse outcomes such as worsening renal function and development of cardiovascular disease. BPis a major promoter of the decline in GFR (16). Alsaedi, Jamal and Al-Windawi (6) Hypertension at the time of diagnosis was found in 11 ADPKD patients (7 males with frequency of 68% vs. 4 females with frequency of 32%). Sahan and Aziz, (17) found Angiotensin *type 1 Receptor* (AT1R) A1166C gene polymorphism, may be associated with hypertension in some Iraqi patients .and the C allele may be considered as a risk factor for developing hypertension.

Clinical characteristic in chronic kidney disease according to Serum urea and Serum creatinine in CKD patients.

Current result revealed that there is significant differences between chronic kidney disease individuals according to blood urea and serum creatinine level, Table (3) shows a significant increase in the level of serum urea and serum creatinine level for CKD patients 136.26±38.80 mol/L, 12.43±6.20 mol/L respectively, compared with the control group 20.58±4.55 mol/L,0.64±0.11 mol/L respectively.

Table (3): Comparison between patients and control for urea blood and Serum creatinine in CKD patients.

	Serum urea (mol\L)		**P value**
CKD patients	136.26±38.80	(63-243)	0.0001#
Controls	20.58±4.55	(11-29)	
	Serum creatinine (mol\L)		
CKD patients	12.43±6.20	(2.30-20.30)	0.0001#
Controls	0.64±0.11	(0.45-0.97)	
-Data were presented as Mean±SD (Range)			
#Significant difference between two independent means using Students-t-test at 0.05 level.			

Increased serum urea levels are common in moderate to advanced chronic kidney disease (CKD). Some studies have shown that urea is a direct and indirect uremic toxin (18). In Indonesia Putra *et al.,* (19) they showed significant mean values for both urea and creatinine serum levels, in Royal Prima General Hospital Medan where decreased levels of urea and creatinine serum for patients after hemodialysis, but still in the abnormal range. Biomarker such as, blood urea, serum creatinine, are routinely used in clinical practice to evaluate renal function. Urea is the main metabolite derived from the turnover of dietary and tissue proteins. The compound is almost exclusively excreted by the kidneys in the urine after filtration in the glomerulus. Several non-renal factors affect serum urea concentration, but reduced urinary removal of urea due CKD is the main

factor in increasing serum urea levels (20).

Distribution of CKD patients and control according to hemoglobin, iron and ferritin

In current research Table (4) shows a significant decrease in the hemoglobin level and iron for CKD patients 9.00±1.84g/dL, 10.17±7.24 Umol/L respectively, compared with the apparently healthy group 13.12±1.75g/dL, 18.80±3.97 respectively. While serum ferritin level result shows a significant increase for CKD patients 506.54±86.16 ng/dL compared with the apparently healthy group126.18±72.56 ng/dL.

These results agree with Kovesdy *et al.*, (21), showed Lower Hb was associated with both significantly higher pre-dialysis mortality and higher risk of ESRD, anemia is associated with both higher mortality and increased risk of ESRD in male patients with CKD not yet on dialysis.

Table (4): Distribution of CKD patients and control according to hemoglobin, iron and ferritin.

	Hemoglobin (g/dL)		**P value**
CKD patients	9.00±1.84	(4.4-12.9)	0.0001#
Controls	13.12±1.75	(9.3-16.3)	
	Iron (Umol\L		
CKD patients	10.17±7.24	(3.4-26.3)	0.0001#
Controls	18.80±3.97	(12.4-27.1)	
	Ferritin (ng\mL)		
CKD patients	506.54±86.16	(387-701)	0.0001#
Controls	126.18±72.56	(21-250)	
-Data were presented as Mean±SD (Range)			
#Significant difference between two independent means using Students-t-test at 0.05 level.			

Hemoglobin (Hb), is the iron-containing oxygen-transport metallo protein in the red blood cells (22). Low hemoglobin (Hb) or anemia, anemia is commonly present in patients with chronic kidney disease (CKD), and is a frequent complication of CKD patients, also hemoglobin levels or the prevalence of anemia increases as kidney function declines (23). Iron deficiency anemia is a common complication of chronic kidney disease (CKD). CKD patients suffer from both absolute and functional iron deficiency. Absolute iron deficiency is defined by severely reduced or absent iron stores, while functional iron deficiency is defined by adequate iron stores (24), slow release of iron from store into circulation is insufficient to compensate the loss due to increased rate of erythropoiesis driven by erythropoietic-stimulating agents (ESAs) (25).

The diagnosis of iron-deficiency anemia in CKD is difficult. The most common biomarkers used to gauge the sufficiency of iron storage are ferritin concentration and transferrin saturation. Both ferritin concentration and transferrin saturation decline in iron-deficiency anemia, ferritin is a positive acute-phase reactant, its concentrations increase in the setting of inflammation. Transferrin, conversely, is a negative acute-phase reactant; its concentrations decrease in patients with inflammation. Accordingly, in an iron-deficient patient, the ferritin concentration may be high and transferrin saturation may be low even in the setting of inflammation (26).

In Table (5), Study correlation between ferritin levels with serum creatinine, hemoglobin and iron in CKD patients, this demonstrated in details below.Ferritin level: There was a highly significant positive correlation with serum creatinine (r=0.430, P=0.002). While a highly significant negative correlation with hemoglobin (r=-0.365, P=0.009) There was also a highly significant negative correlation with iron (r=-0.409, P=0.003).

Table (5): Correlation between ferritin level with serum creatinine, hemoglobin and iron in CKD patients.

CKD patients (n=50)		Ferritin (ng\mL)
Serum creatinine (mol\L)	r	0.430**
	P	0.002
Hemoglobin (g/dL)	r	-0.365**
	P	0.009
Iron (Umol\L)	r	-0.409**
	P	0.003
***Correlation is significant at the 0.05 level. **Correlation is highly significant at the 0.01 level.. correlation(r),**		

Allelic discrimination of rs2070744 C>T polymorphism in NOS3 gene

Distribution of genotypes and allele frequency in chronic kidney disease Iraqi patients and apparently healthy individuals presented in Figure (1).

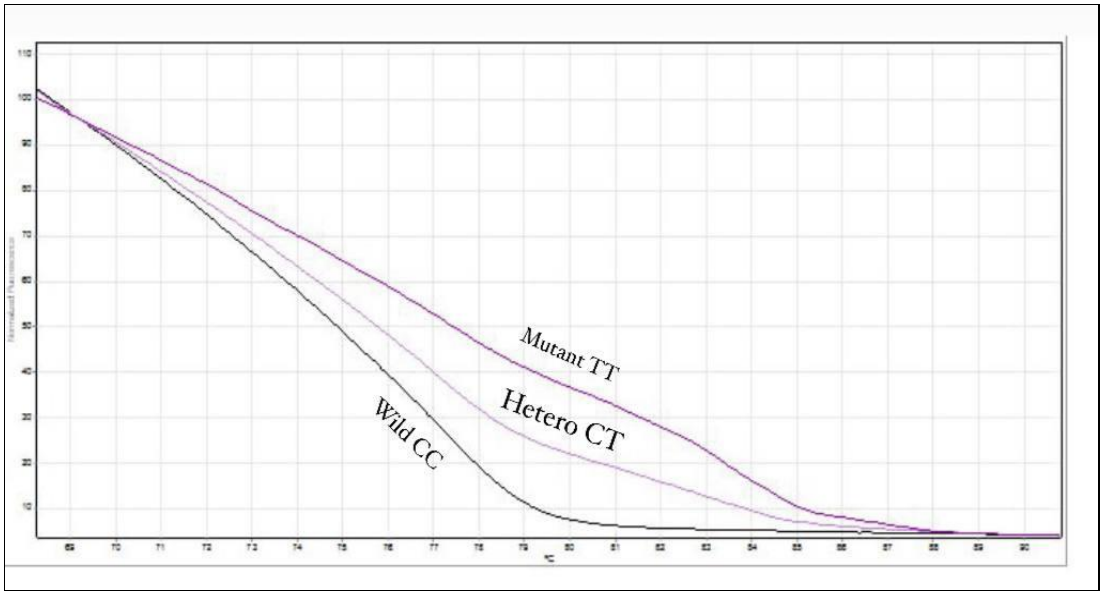

Figure (1): Shows the allelic discrimination of *rs2070744* in *NOS3*.

Table (6) revealed the genotype and allele frequency of rs2070744 SNP at Nos3 gene in control groups versus with CKD patients. The percentage of TT genotype was in CKD patients significantly than that of control groups (42% versus 6%, respectively. While the percentage of CT genotype was significantly in CKD patients than in control groups (21% versus 46%, respectively). There was no significant difference in CC genotype percentage

between control groups and CKD patients as related with rs2070744 at Nos3gene. In this study, it was found that the TT and CT genotype there is significant statistically at $p \leq 0.05$ between patients and control groups at threefold (OR, 21.0,95% CI 4.92-89.56), (OR, 2.74,95% CI 1.01-7.41), respectively. The T allele frequency values were 63% and 29% for CKD patients and control groups. Also, C allele frequency values were 37% and 71% for CKD patients and control groups, respectively table.

Table (6): Comparison of the Genotype and Allele Frequencies of *Nos3* gene polymorphism (*rs* 2070744 C>T) between Patients Group and Control group

		CKD patients		Controls		P value	OR (95%CI)
		No.	%	No.	%		
***Nos3gene* rs (2070744 C>T)**	**TT (Mutant)**	21	42.0	3	6.0	0.0001*	21.0(4.92-89.56)
	CT (Hetero)	21	42.0	23	46.0	0.044*	2.74(1.01-7.41)
	CC (Wild)	8	16.0	24	48.0		---
Allele Frequency	**T**	63	63%	29	29%	0.0001	4.16(2.30-7.54)
	C	37	37%	71	71%		
-Odds Ratio & its 95% Confidence interval							
*Significant difference between percentages using Pearson Chi-square test (χ^2-test) at 0.05 level.							

All three isoforms of nitric oxide synthase are expressed in renal glomeruli. In the renal glomerulus, nitric oxide plays an important role in the homeostasis of sodium and water, contributing to the maintenance of normal vascular tone, regulating the – natriuretic response and tubule glomerular feedback of normal pressure, inhibiting tubular reabsorption of sodium, and regulating sympathetic activity. As expected, NOS3 is expressed primarily in renal vascular endothelial cells, including afferent and efferent arteries (27). Asp298Glu polymorphism in NOS3 is already associated with endothelial dysfunction due to the destruction of endothelial cells. Many diseases are occur one of the disease is CKD (28). In Chronic kidney disease or non-traditional etiology (CKDnT) the rs2070744 polymorphism, the CC genotype was more common in patients than in controls This result were disagree with a current study due to variation of the genotype in individuals a cross the world. Chronic kidney disease of uncertain or non-traditional etiology (CKDnT) is a term that has been used to describe CKD that is not attributable to any traditional risk factor and is characterized by rapid progression (29) this disease spreading in Mexico and Central America has etiology a strong environmental component, and perhaps a genetic susceptibility De Silva *et al*., (30). This disease has been related to the use of pesticides, recurrent episodes of dehydration and changes in the intestinal microbiota, among other environmental factors (31).

Marín *et al*., (32) found in the CKDnT the rs2070744 polymorphism, the CC genotype was more common in patients than in controls This result were disagree with a current study due to variation of the genotype in individuals a cross the world. Nitric oxide synthesis 3 (Nos3) gene rs2070744 polymorphism found to be associated with diabetic nephropathy in different studies Medina *et al*., (2). In the *NOS3* gene, polymorphism (*rs*

2070744 C>T) located in the promoter of the gene, this polymorphism have been associated with many diseases such as atherosclerosis, coronary spasm induced by acetylcholine, hypertension, Alzheimer's disease, hypertensive disease of pregnancy and prostate cancer (33).

Conclusion

The current research the effect of the variant allele for SNPs (rs2070744C>T) of NOS3 gene appears to be related with CKdisease, suggest to have a modifier effect on disease incidence.

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