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Original Research Article

Association of Endothelial Nitric Oxide Synthase (eNOS) Gene Polymorphism and Chronic Kidney Disease in Maiduguri, Borno State, Nigeria

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Abstract: Chronic kidney disease (CKD) is becoming an important health problem worldwide. Since genetic risk factor is implicated in CKD development, this study aimed at assessing association of eNOS gene polymorphism and chronic kidney disease in Maiduguri. One hundred and forty (140) CKD patients and 70 apparently healthy controls participated in this study. Five milliliters (5ml) of whole blood were taken from each participant and sent to genetic laboratory. DNA was extracted from whole blood samples using GDSBio Blood Genomic DNA extraction kits. Then PCR and Agarose gel electrophoresis was performed to genotype Glu298 ASP, VNTR in intron4 and T786C variant of eNOS gene using special primers for three genes. In this study CKD patients had an average age of 49.51±16.7 years compared to 45.65±16.70 years for controls. Also 82 (58.57%) of CKD patients were hypertensive as compared to 26 (37.14%) control subjects. This study demonstrated significant association between eNOS gene polymorphism Glu298 ASP (P=0.000), VNTR in intron4 (P=0.000), T786C (P=0.005) and CKD. The study found significant associations between eNOS gene polymorphisms and CKD. These findings may guide clinical interventions aimed at slowing CKD progression, including the management of genetic risk factors.

Keywords: Gene, Kidney Disease, Polymorphism, Endothelium, Synthase, Nitric Oxide.

INTRODUCTION

Chronic kidney disease (CKD) is a multifactorial disease associated with both environmental and genetic components. It is an irreversible process that often leads to a terminal state in which the patient requires renal replacement therapy (Medina *et al*., 2018). Most cases of CKD are due to chronic-degenerative diseases and endothelial dysfunction is one of the factors that contribute to its pathophysiology. One of the most important mechanisms for proper functioning of the endothelium is the regulation of the synthesis of nitric oxide (NO). This compound is synthesized by the enzyme nitric oxide synthase (NOS), which has 3 isoforms: neuronal NOS (NOS1), inducible NOS (NOS2) and endothelial NOS (eNOS or NOS3) (Sahar and Soha, 2011; Gao *et al*., 2017) among which eNOS plays a key role in the regulation of vascular function (Yanming *et al*., 2011). The human eNOS, is a multi-domain enzyme that uses several cofactors including tetrahydrobiopterin (BH4), nicotinamide-adenine -dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN). Proteolytic activity at the calmodulin-binding site is able to cleave the protein into C-terminal region and N-terminal region. The C-terminal region contains FMN, FAD and reduced form of nicotinamide-adenine dinucleotide phosphate (NADPH)-binding regions, whereas the N-terminal region contains the oxygenase fragment and binding site for L-arginine, tetrahydrobiopterin (BH4) and heme (Medina *et al*., 2018). The eNOS gene is located on chromosome 7q35–36 and comprises 26 exons and 25 Intron that span 21 kb and is expressed mainly in the endothelium. It encodes for eNOS, an enzyme composed of 1203 amino acids with a molecular weight of 133 289 Da. The enzyme acts as a homodimer and is located in

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the cell membrane, cytoplasm, and Golgi apparatus (Medina *et al*., 2018). eNOS has Vaso-protective effects by scavenging superoxide radicals and suppressing platelet aggregation, leucocyte adhesion and smooth muscle cell proliferation (Yanming *et al*., 2011). The activity of the enzyme is regulated by free calcium and the subsequent union of the calcium-calmodulin complex. Several allelic variants of the eNOS gene may affect NO production. Three eNOS polymorphisms, the Intron 4 27-bp repeat (4b/a), the G894T (Glu298Asp; codon GAG to GAC) missense mutation in exon 7 and the T786C single-nucleotide polymorphism in the promoter region have been studied. T786C reduces eNOS transcription; G894T may be associated with a decrease of eNOS activity and 4b/a polymorphism has been reported to be associated with decreased plasma nitric oxide (NO) concentrations that may reflect the activity of eNOS (Yanming *et al*., 2011).

Polymorphisms in the NOS3 gene have been implicated as factors that alter the homeostasis of this mechanism. The Glu298Asp polymorphisms, VNTR in Intron4 (4 b/a) and -786T>C of the NOS3 gene have been associated with a more rapid deterioration of kidney function in patients with CKD (Medina *et al*., 2018). Alterations in endothelial-derived Nitric Oxide (NO) production have been associated with numerous diseases and in humans, can be genetically determined by the presence of different polymorphisms in the eNOS gene.

Nitric Oxide and Renal Endothelial Dysfunction

One of the factors that regulate vascular tone and influence endothelial dysfunction is nitric oxide (NO). This compound is synthesized in the vascular endothelium by the action of the enzyme nitric oxide synthase (NOS) which catalyzes the conversion of L -arginine to L -Citrulline generating NO as a by-product. It is a gas with half-life of 0.5 to 5 seconds that easily disseminates from the endothelial cells to the smooth muscle cells of the vascular wall (Sahar and Soha, 2011; Medina *et al*., 2018) and it is the main vasodilator at the renal level (Marin-Medina *et al*., 2023). The released NO mediates local vasodilatation and inhibits platelet aggregation, leukocyte adhesion, and vascular smooth muscle cell proliferation (Gao *et al*., 2017). In the kidney, NO is involved in the regulation of renal plasma flow, GFR, sodium excretion, extracellular fluid volume, and the maintenance of renal structural integrity. The important role of NO in the regulation of the hemodynamic and metabolic milieu in the kidney suggests that an abnormal eNOS activity due to a genetic mutation could be implicated and may therefore aggravate renovascular injury (Medina *et al.,* 2018). Endothelial dysfunction and impaired regulation of nitric oxide (NO) system has emerged as the characteristics of chronic kidney diseases (CKD)(Ilhan *et al*., 2016).

MATERIAL AND METHODS

Study Site and Sampling

This study is a cross-sectional, multicenter in which 140 eligible and clinically confirmed CKD patients between the age of 19 and 90 years were recruited from nephrology clinics of University of Maiduguri Teaching Hospital (UMTH) and State Specialist Hospital Maiduguri (SSHM) Borno state, from February to April 2022. During the same period, 70 controls were recruited from same health Care centers.

Ethical Considerations

Before commencement of sample collection, an ethical approval was obtained from the Ethical Research Committee of UMTH with reference number ADM/TH/497/VOL.1of 21st June, 2023 and Borno State Ministry of Health, in accordance with Helsinki declaration with reference number SHREC Approval No.11/2023.

Informed Consent

Eligible individual subjects were duly informed about the research work and consent forms signed by the patients. Following informed consent, a structured interview-administered questionnaire was used to obtain data on subject's demographic variables, comorbid disease to CKD, social life style and anthropometric variables.

BLOOD COLLECTION AND PROCESSING

Five milliliters (5mL) of blood sample were collected from each participant and dispensed into labelled ethylene diamine tetra-acetic acid (EDTA) bottle. The whole blood in the EDTA container was stored at -80^{o}C until analysis.

DNA Extraction and Genotyping

Genomic DNA was extracted from leukocytes in the buffy coat separated from whole blood samples (CKD patients n = 140 and Control subjects n= 70) by using GDSBio Blood Genomic DNA Extraction Kit according to the manufacturers' protocol. Isolated DNA concentration and purity was determined using NanoDrop 2000/2000C (Thermo Scientific, Boston, MA, USA). Polymerase chain reaction (PCR) amplification followed by direct agarose gel electrophoresis was used to genotype variable number of tandem repeats (VNTR) intron 4 (27 bp), while RFLP was used to genotype T786C (rs2070744) and G894T(rs1799983) variants with sequence-specific primers as previously described by (Luizon *et al*., 2009).

Genotyping of VNTR 27 bp Intron 4 of eNOS Gene

The extracted DNA was amplified for polymorphic VNTR in intron 4 by PCR with a standard protocol and previously published primers(Luizon *et al*., 2009). Primer sequences 5′-AGG GGG TAT GGT AGT GCC TTT-3′ (forward) and 5′-TCT CTT AGT GCT GTG GTC AC-3′ (reverse) were used for the PCR amplification. PCR was performed for 35 cycles in

thermocycler (Eppendorf Master Cycler Nexus, Hamburg, Germany). The amplification conditions were the following: initial denaturation 95ºC for 3 min, denaturation 95 ºC for 30 seconds, annealing at 54ºC for 30 seconds, extension at 72ºC for 30 seconds, for 35 cycles and final extension 72ºC for 5 min. The products were then resolved on 2.0% agarose gel for VNTR variants; 4a, 4b. A fragment of 420 bp in the case of allele b indicates genotype 4b4b, and a fragment of 393 bp in case of allele a indicates genotype 4a4a and a Fragment of 420bp and 393bp indicates genotype 4b4a and they were estimated with a 25 bp DNA ladder (New England Biolabs Inc., Ipswich, MA, USA).

Genotyping of T786C (rs2070744) Variants in eNOS Gene Promoter Region

PCR-RFLP was used to genotype the T786C (rs2070744) variation with the primer pair 5' -TGGAG AGTGCTGGTGTACCCCA-3'(forward) and 5' - GCCTCCACCC CCACCCTGTC-3' adopted from previously published primer.[9] PCR was performed for 35 cycles in thermocycler (Eppendorf Master Cycler Nexus, Hamburg, Germany). The amplification conditions were the following: initial denaturation 95ºC for 3 min, denaturation 95 ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 30 seconds, for 35 cycles and final extension 72ºC for 5 min. A 180bp fragment amplified was digested with the restriction enzyme Msp I. Cuts of 140 bp and 40bp were identified in the case of the T allele and 90 bp, 50 bp and 40 bp in the case of the C allele when agarose gel images were visualized on the agarose gel images photographed.

Genotyping of G894T (rs1799983) Variants in eNOS Gene

PCR-RFLP was used to genotype the G894T (rs1799983) variation with the primer pair 5' - AAGGCAGGAGACAGTGGATGGA-3' (forward) and 5' - CCCAGTCAATCCCTTTGGTGCTCA-3'adopted from previously published primer.[9] PCR was performed for 35 cycles in thermocycler (Eppendorf Master Cycler Nexus, Hamburg, Germany). The amplification conditions were the following: initial denaturation 95ºC for 3 min, denaturation 95 ºC for 30 seconds, annealing at 60ºC for 30 seconds, extension at 72ºC for 30 seconds, for 35 cycles and final extension 72ºC for 5 min. A 248-base pair (bp) fragment amplified was subjected to digestion with Ban II enzyme, which produces a fragment of 163 and 85 bp in the case of the G allele, and no cut occurs (248 bp) in the case of the T allele when agarose gel images were visualized on the agarose gel images photographed.

Statistical Analysis

All data collected were fed into a computer and analyzed using the Statistical Package for Social Sciences (SPSS) for Windows software version 25.0. Categorical variables are expressed as frequencies (%) and continuous variables as mean and standard deviation (SD) of normally distributed data. The comparison of genotype distributions of eNOS gene variants between groups was analyzed with the Pearson chi-square test. A p value equal to or less than 0.05 were taken as statistically significant.

RESULTS

Table 1 provides general characteristics of the studied population. It provides demographic, lifestyle, family history, and health factors between the CKD patients and controls. The study revealed CKD patients had an average age of 49.51±16.7 years compared to 45.65±16.70 years for controls. The demographic characteristics of the study population showed males were found more 88(62.85%) in the CKD patients and females more 39(55.71%) in the controls. On the other hand, cigarette smoking was found to be low among the overall study population but slightly higher in CKD patients (7.14%) compared to the controls (2.86%). Also 28 (20%) of CKD patients had family history of kidney disease as compared to 7(10%) of the controls. This study also observed that 18.57% of CKD patients had family history of diabetes mellitus compared to 11.43% of controls. Also 82 (58.57%) of the CKD patients were found to be hypertensive as compared to 26 (37.14%) control subjects who were hypertensive. The Mean ±SD of systolic blood pressure (SBP) and diastolic blood pressure (DBP) of CKD patients were 137.72±27.27mmHg and 88.39±19.68mmHg respectively as compared to control groups who had Mean ±SD of SBP and DBP of 127.61±17.85mmHg and 84.23±11.21mmHg respectively.

Table 1: General characteristics of the population studied

Variables	Subjects	
	CKD patients n(%)	Controls p-values n(%)
Sex		
Female	52(37.15)	39(55.71)
Male	88(62.85)	31(44.29)
Family History of Kidney Disease		
Yes	28(20.00)	7(10.00)
No	112(80.00)	63(90.00)
Family History of DM		
Yes	26(18.57)	8(11.43)
No	114(81.43)	62(88.57)

Hypertension		
Yes	82(58.57)	26(37.14)
No	58(41.43)	44(62.86)
Blood Pressure	**Mean±SD**	
SBP(mmHg)	137.72±27.27	127.61±17.85
DBP(mmHg)	88.39±19.68	84.23±11.21
Age in Years(Mean±SD)	49.51±16.7	45.65±16.70
Anthropometrics Measures Mean±SD		
Ht (m)	1.97±0.18	1.64±0.07 0.539
Wt (kg)	61.60±15.98	64.70±19.28 0.573
WC(cm)	36.02±5.0	37.56±9.39 0.983
BMI(Kg/m^2	22.29±6.49	22.70±6.67 0.704

Key: SBP=Systolic DBP=Diastolic Blood Pressure Ht= Height, Wt=Weight, WC= Waist Circumference Blood pressure

In this study we genotyped eNOS gene polymorphism in 140 CKD patients and 70 Control subjects as shown in table 2. The result depicts frequencies of the three variants of the eNOS gene that is rs1799983, Intron4 VNTR and rs2070744. The rs1799983 (G894T) or Glu298Asp polymorphism shows that the GG genotype was observed more frequently in the control subjects than in the CKD patients (77.14% vs 35.71%) and the difference was found to be statistically significant while the heterozygous GT and homozygous TT genotypes were more frequent in the CKD patients than in the control subjects (50.00% vs 17.14% and 14.29% vs 5.72%) respectively and the difference were statistically significant(p=0.000). In the case of Intron4 VNTR polymorphism, the result showed frequencies of the genotype 4b/a in the study subjects. Genotype 4bb showed high frequencies in the control subjects (74.29%) than in the CKD patients (42.86%). However, heterozygous genotype 4a4b and homozygous 4a4a were found to have high frequencies in CKD patients than in the control subjects (48.57% vs 20.00%) and (8.57% vs 5.71%) respectively. The difference in the genotypes between the two groups was found to be statistically significant (p=0.000). Our study also observed frequencies of genotype in rs2070744 (T786C) polymorphism. The study observed high frequency of TT genotype in control subjects than in CKD patients (71.43% vs 48.575%). Heterozygous genotype TC was observed with high frequency in CKD patients than in control subjects (44.29% vs 21.43%) and the difference was found to be statistically significant(p=0.005). However, genotype CC was observed with equal frequencies between the two groups.

Table 2: Genotype distribution of the three eNOS gene polymorphism among patients and control groups

Genotypes	CKD Patients n(%)	Control subjects n(%)	Chi Square
Rs1799983 (G894T)			
GG	50(35.71)	54(77.14)	
GT	70(50.00)	12(17.14)	X^2=31.595
TT	20(14.29)	4(5.72)	P=0.000
Total	**140(100)**	**70(100)**	
Intron 4 VNTR (4b/a)			
bb	60(42.86)	52(74.29)	X^2=18.485
ab	68(48.57)	14(20.00)	P=0.000
aa	12(8.57)	4(5.71)	
Total	**140(100)**	**70(100)**	
Rs 207074 (T786C)			
TT	68(48.57)	50(71.43)	X^2=10.634
TC	62(44.29)	15(21.43)	P=0.005
CC	10(7.14)	5(7.14)	
Total	**140(100)**	**70(100)**	

Key: G=guanine, T=thymine, C=Cytosine, a= a allele of eNOS intron 4 a/b VNTR polymorphism b= b allele of eNOS intron 4 a/b VNTR polymorphism, CKD= chronic kidney disease

Table 3-6 Relationship between genotype of eNOS gene and mean concentration of cadmium of CKD patients and control subjects.

Table 3 shows the relationship between mean urinary concentrations of cadmium in CKD patients and control subjects with respect to eNOS gene genotype. The result indicated high concentrations of the Cadmium in urine of CKD patients with respect to eNOS gene genotypes of 4b4b, 4a4b,4a4a (VNTR); GG, GT, TT(G894T) and TT, TC, CC(T786C) compared to the control subjects. However, there was no significant

(P>0.05) difference between the two groups with respect to the eNOS gene genotypes.

Table 3: Relationship between genotype of eNOS gene and mean concentration of cadmium of CKD patients and control subjects

Genotype	Mean ± SE of cadmium		P-Value
	CKD Patient	CONTROL Subjects	
VNTR 4b4b	0.09±0.08	0.08±0.02	0.745*
4a4b	0.11±0.06	0.06±0.03	
4a4a	0.07±0.03	0.03±0.01	
G894T GG	0.09±0.01	0.07±0.01	0.956*
GT	0.12±0.01	0.07±0.04	
TT	0.06±0.02	0.03±0.01	
T786C TT	0.10±0.01	0.07±0.02	0.783*
TC	0.07±0.01	0.08±0.02	
CC	0.05±0.02	0.04±0.01	

Key: *NS (Not significant) p> 0.05, G=guanine, T=thymine, C=Cytosine, a= a allele of eNOS intron 4 a/b VNTR polymorphism, b= b allele of eNOS intron 4 a/b VNTR polymorphism, CKD= chronic kidney disease

Similarly, table 4 shows the relationship between mean urinary concentrations of mercury in CKD patients and control subjects with respect to eNOS gene genotype. The result indicated high concentrations of the mercury in urine of CKD patients with respect to eNOS gene genotypes of 4b4b, 4a4b,4a4a (VNTR); GG, GT, TT(G894T) and TT, TC, CC(T786C) compared to the control subjects. The result also shows no significant (P>0.05) difference between the two groups with respect to the eNOS gene genotypes.

Table 4: Relationship between genotype of eNOS gene and mean concentration of Mercury of CKD patients and control subjects

Genotype	Mean ± SE of Mercury		P-Value
	CKD Patient	CONTROL Subjects	
VNTR 4b4b	0.35±0.13	0.34±0.13	0.352*
4a4b	0.39±0.19	0.39±0.05	
4a4a	0.42±0.14	0.33±0.08	
G894T GG	0.36±0.02	0.36±0.02	0.795*
GT	0.38±0.02	0.29±0.03	
TT	0.37±0.04	0.35±0.08	
T786C TT	0.39±0.02	0.36±0.02	0.232*
TC	0.36±0.01	0.33±0.03	
CC	0.30±0.01	0.36±0.05	

Key: *NS (Not significant) p> 0.05, Key: G=guanine, T=thymine, C=Cytosine, a= a allele of eNOS intron 4 a/b VNTR polymorphism, b= b allele of eNOS intron 4 a/b VNTR polymorphism, CKD= chronic kidney disease

Table 5 also shows the relationship between mean urinary concentration of Lead (Pb) in CKD patients and control subjects with respect to eNOS gene genotype. The result indicated high concentrations of Pb in urine of CKD patients with respect to eNOS gene genotypes of 4b4b, 4a4b,4a4a (VNTR); GG, GT, TT(G894T) and TT, TC, CC(T786C) compared to the control subjects. There was also no significant (P>0.05) difference between the two groups with respect to the eNOS gene genotypes.

Table 5: Relationship between genotype of eNOS gene and mean concentration of Lead of CKD patients and control subjects

Genotype	Mean ± SE of Lead		P-Value
	CKD Patient	CONTROL Subjects	
VNTR 4b4b	0.04±0.01	0.03±0.004	0.271*
4a4b	0.02±0.02	0.02±0.002	
4a4a	0.03±0.02	0.03±0.02	
G894T GG	0.03±0.003	0.02±0.004	0.980*
GT	0.02±0.002	0.01±0.003	
TT	0.02±0.004	0.01±0.003	
T786C TT	0.02±0.002	0.01±0.004	0.517*
TC	0.02±0.003	0.04±0.01	

Genotype	Mean ± SE of Lead		P-Value
	CKD Patient	CONTROL Subjects	
CC	0.03±0.01	0.01±0.01	

Key: *NS (Not significant) p> 0.05, Key: G=guanine, T=thymine, C=Cytosine, a= a allele of eNOS intron 4 a/b VNTR polymorphism, b= b allele of eNOS intron 4 a/b VNTR polymorphism, CKD= chronic kidney disease

However, in table 6 the concentration of the heavy metal Arsenate was high in CKD patient than the control subjects. The result indicates that there was significant(p=0.023) difference in mean urinary Arsenate concentration in CKD patients only with respect to VNTR genotypes(4b4b,4a4b,4a4a) as compared to the control subjects.

Table 6: Relationship between genotype of eNOS gene and mean concentration of Arsenic of CKD patients and control subjects

Genotype	Mean ± SE of Arsenic		P-value
	CKD Patient	CONTROL Subject	
VNTR 4b4b	0.04±0.01	0.02±0.01	0.023**
4a4b	0.03±0.01	0.02±0.01	
4a4a	0.06±0.01	0.02±0.02	
G894T GG	0.02±0.001	0.01±0.003	0.616*
GT	0.03±0.001	0.02±0.01	
TT	0.02±0.002	0.01±0.01	
T786C TT	0.03±0.002	0.02±0.004	0.739*
TC	0.04±0.002	0.02±0.01	
CC	0.01±0.003	0.01±0.001	

Key: *NS (Not significant) p> 0.05, **S(Significant) P<0.05, Key: G=guanine, T=thymine, C=Cytosine, a= a allele of eNOS intron 4 a/b VNTR polymorphism, b= b allele of eNOS intron 4 a/b VNTR polymorphism, CKD= chronic kidney disease

Figure 1 shows amplified 393 bp and/or 420 bp segment of the eNOS intron 4 VNTR region. eNO3 intron 4 VNTR genotypic distributions were determined as 393 bp, 393bp and 420 bp, and 420 bp for a/a, a/b, and b/b genotypes, respectively. The result shows Lane L is 25bp DNA Ladder, Lane H1, H5 and H8 are 393bp each; Lane H4 and H7 are 420bp; while Lane H2, H3, H9, H10 and H11 are 420bp and 393bp each. 420bp (A fragment of 420 bp was produced in the case of allele b (indicates genotype 4b4b), and a fragment of 393 bp in case of allele a (indicates genotype 4a4a) and a fragment of 420bp and 393bp (indicates genotype 4b4a)

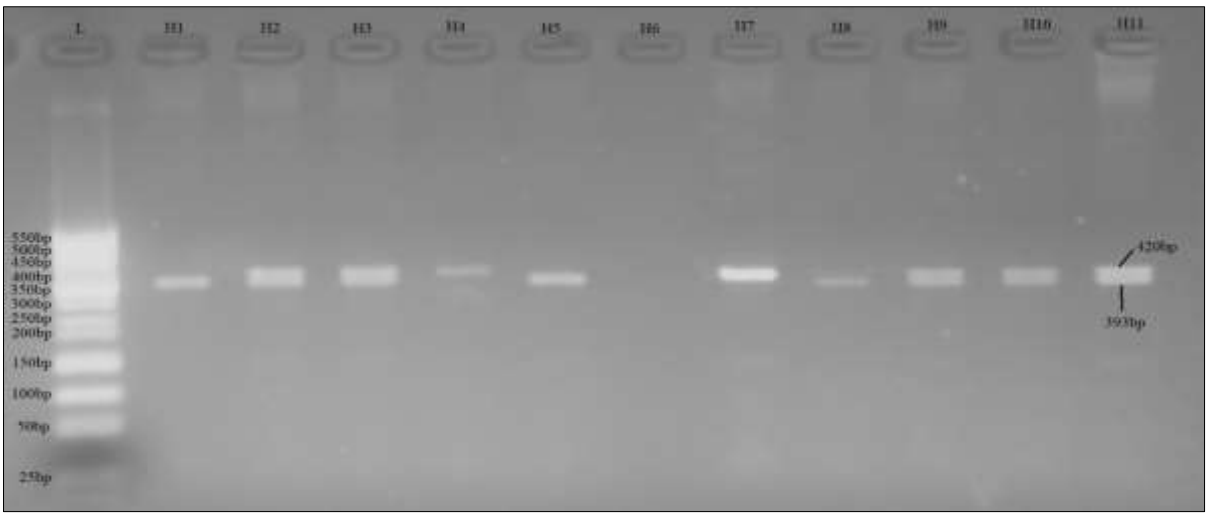

Figure 1: A Representative of PCR Agarose Gel Electrophoresis for VNTR eNOS gene Polymorphism. Lane L is 25bp DNA Ladder. Lane H1, H5 and H8 are 393bp each; Lane H4 and H7 are 420bp while Lane H2, H3, H9, H10 and H11 are 420bp and 393bp each

Figure 4.2 shows the result of digestion of PCR amplicon of (G894T) eNOS gene with BanII restriction enzyme. The result shows PCR products of 248bp, 163bp and 85bp fragments. Lane L is Norgen minisizer 25bp DNA Ladder, Lane H2, H4, H7 and H8 are 163bp fragments while Lane H6 has 248, 163 and 85bp fragments. Fragment of 163 and 85 bp (indicate the case of G allele, and no cut (248bp) in the case of T allele. The

GG genotype (wild-type) showed a single band at 248 bp (base pair). The TT genotype (homozygous mutation) showed two bands at 163 and 85 bp. The GT genotype (heterozygous mutation) showed three bands at 248, 163, and 85 bp.

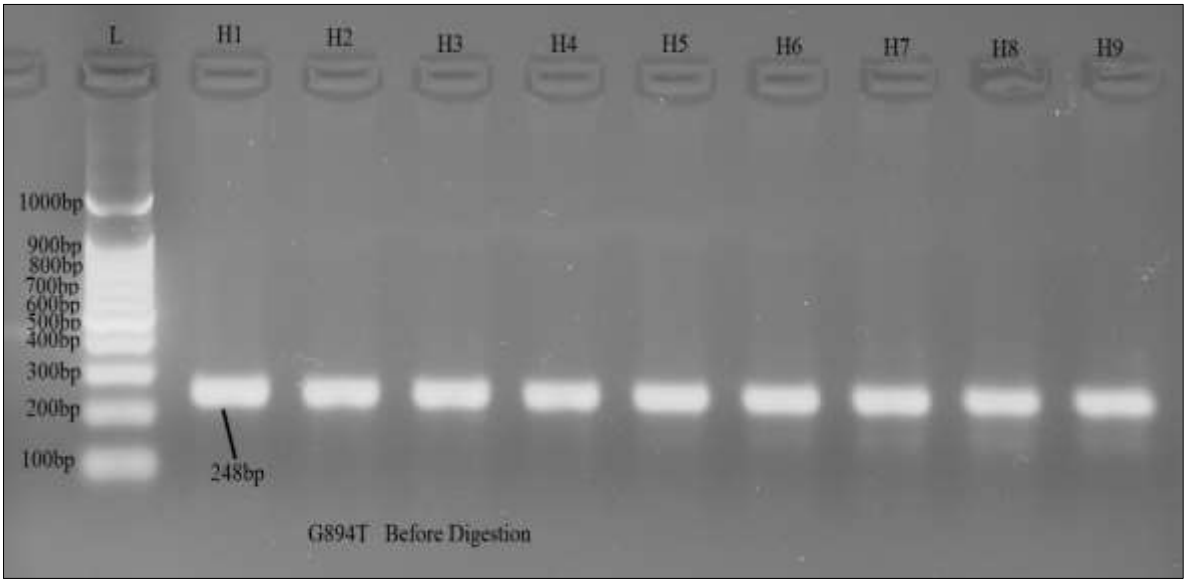

Figure 2: Shows a representative PCR Agarose Gel Electrophoresis of 10 samples H1 to H10 for G894T eNOS gene polymorphism of size 248bp before digestion using a ladder L of 100bp

Figure 3 shows the result of digestion of PCR amplicon of (G894T) eNOS gene with BanII restriction enzyme. The result shows PCR products of 248bp, 163bp and 85bp fragments. Lane L is Norgen minisizer 25bp DNA Ladder, Lane H2, H4, H7 and H8 are 163bp fragments while Lane H6 has 248, 163 and 85bp fragments. Fragment of 163bp and 85 bp (indicate the case of G allele, and no cut (248bp) in the case of the T allele. The GG genotype (wild-type) showed a single band at 248 bp. The TT genotype (homozygous mutation) showed two bands at 163bp and 85 bp. The GT genotype (heterozygous mutation) showed three bands at 248bp, 163bp, and 85 bp.

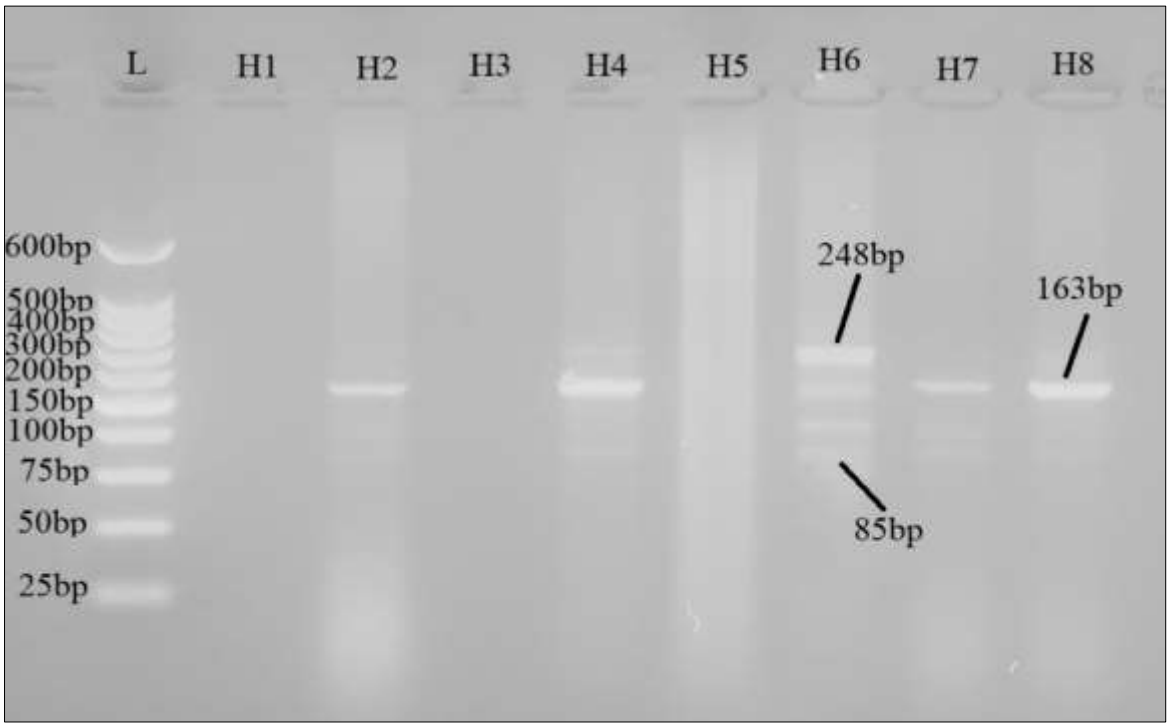

Figure 3: A Representative of PCR Agarose Gel Electrophoresis for G894T eNOS gene polymorphism after digestion with BanII restriction enzyme

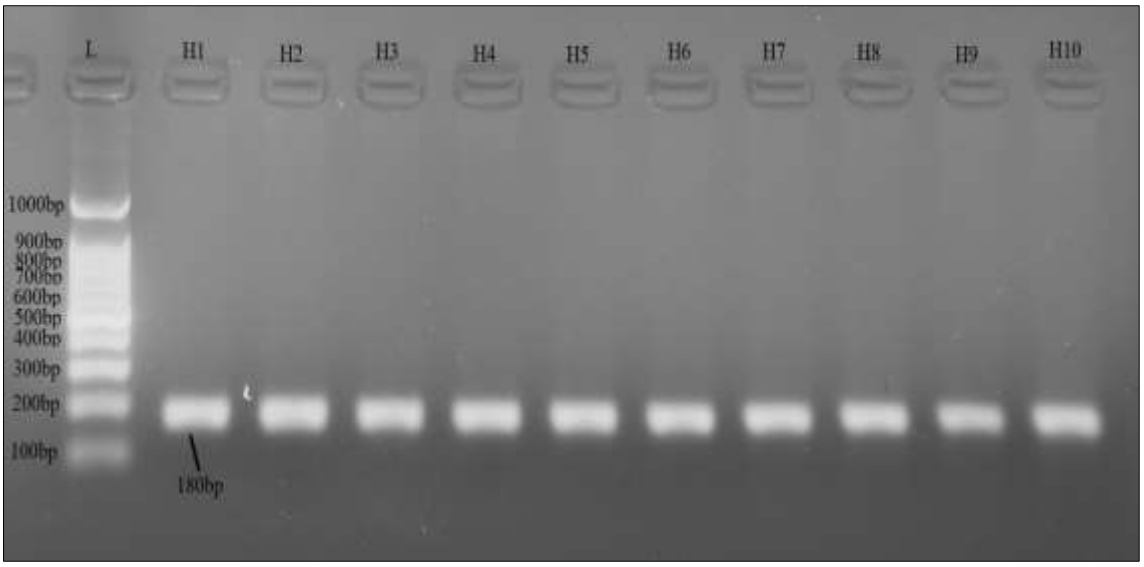

Figure 4: Shows a representative PCR Agarose Gel Electrophoresis of 10 samples H1 to H10 for T786C eNOS gene polymorphism of size 180bp before digestion using a ladder L of 100bp.

Figure 5 shows the result of digestion of PCR amplicon 0f (T786C) eNOS gene with Msp1 restriction enzyme. The result shows PCR products of 140bp, 90bp,50bp and 40bp fragments. Lane L is 25bpDNA Ladder, Lane LD is Lambda DNA, Lane H, H3, H6, H7, H10, H11, and H12 are 90bp and 50bp fragments each. Lane H2, and H4 have 140bp,90bp, and 50bp fragments each while Lane H5, H8 and H9 are 90bp,50bp and 40bp fragments each. Fragment of 140 and 40bp identified in the case of wild type(T) allele and fragments of 90, 50 and 40 bp identified in the case of the variant (C) allele.

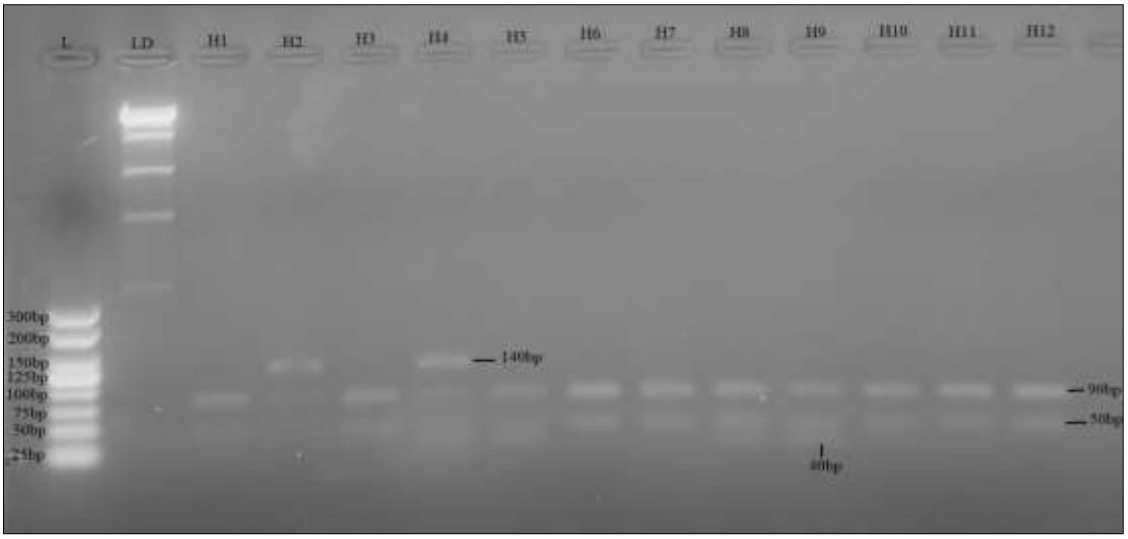

Figure 5: A Representative of PCR Agarose Gel Electrophoresis for T786C eNOS gene polymorphism after digestion with Msp1 restriction enzyme

DISCUSSION

Chronic kidney disease (CKD) is recognized as a global public health problem. CKD is a multifactorial disease with associated genetic and environmental risk factors (Cañadas-Garre *et al*., 2018. It is characterized by structural and functional abnormalities of the kidney that often progresses to end-stage renal failure (ESRF)(Buddhi *et al*., 2019). The two major causes of CKD world-wide are the traditional etiologies Hypertension (HTN) and Diabetes mellitus (DM). However, chronic glomerulonephritis, interstitial nephritis and Sickle cell disease are also a major cause of CKD in developing countries. The prevalence of CKD is also increasing at a more rapid rate in developing countries (Medina *et al*., 2018). Because of this, there is the need to identify CKD patients at earlier stages so as to improve stratification of their risk for progression to end-stage renal disease (ESRD). Therefore, this study aimed at identifying other causes such as environmental (heavy metals) and genetic susceptibility to CKD and early Biomarkers of renal disease. Therefore, we recruited 140 clinically confirmed CKD patient and 70

apparently healthy subjects as control who have met our inclusion criteria. The CKD patients consist of 88(62.85%) males and 52(32.15%) females. Similarly, the control subjects consist of 31(44.29%) male and 39(55.71%) females age matched with the CKD patients. The average ages of the CKD patients were 49.51±16.7 years (range 19-90years) and that of the control was 45.65±16.70years (range 14-90years).

This study investigated eNOS gene polymorphism in the study population in order to establish genetic susceptibility to CKD in the study area. Several studies have reported association between allelic variant of eNOS gene and nitric oxide (NO) production. NO has a crucial role in vascular homeostasis, regulation of blood flow and blood pressure (Ilhan *et al*., 2016). It also mediates local vasodilatation and inhibition of platelet aggregation, leukocyte adhesion and vascular smooth muscle proliferation. Its major role in renal homeostasis involves regulation of renal plasma flow, glomerular filtration rate, sodium excretion, extracellular fluid volume and maintenance of renal structural integrity (Sahr and Soha, 2011). NO is produced in vascular endothelial cells by endothelial nitric oxide synthase (eNOS) enzyme. eNOS enzyme catalyzes the conversion of L-arginine to NO and L-Citruline as a by-product. Therefore, eNOS polymorphism are found to be associated with decreased enzyme activity of eNOS and thus decreased basal NO production. These effects also have been found to cause increased circulating NOS inhibitors like Asymmetry Dimethyl arginine (ADMA) which contributes to an arterial pressure or intraglomerular hypertension in CKD patients(Ilhan *et al*.,2016). Although there are conflicting data in the literature concerning association of eNOS gene polymorphism and CKD and its progression to ESRD, our study has found significant association between eNOS polymorphism and CKD. This present study demonstrated polymorphism in the G894T SNP (rs1799983) of the NOS3(Glu298Asp variant) gene in the study subjects. This study demonstrated heterozygous GT and homozygous TT genotypes were more frequent in the CKD patients than in the control group 50.00% vs 17.14% and 14.29% vs 5.72% respectively and the difference were statistically significant(p=0.000). However, GG genotype was observed more frequently in the control group than in the CKD patients (77.14% vs 35.71%) and the difference was found to be statistically significant(P=0.000). This finding is in agreement with report of Marin-Medina *et al*., 2023. This SNP affects the oxidase domain of the enzyme thereby alters the stability, biological half-life, and activity of the enzyme and thus is associated with reduced NO (Wang *et al*., 1997). The polymorphisms in the NOS3 gene have been found to be associated with endothelial dysfunction in different populations (Medina *et al*., 2018). In the case of intron4 VNTR polymorphism, this study observed that heterozygous genotype 4a4b and homozygous 4a4a were found with high frequency in CKD patients than in control group 48.57% vs 20.00% and 8.57% vs 5.71% respectively. The difference in the genotype between the two group was found to be statistically significant(p=0.000). However, the genotype 4bbwas observed with high frequency in control group than in CKD patients. This observation was in agreement with a report of Zhu *et al*., (2014) who reported a significantly high frequency of 4a allele in eNOS Intron4 in CKD patients than in controls. They also reported higher frequencies of carrier genotypes eNOS 4aa and 4ab in CKD patients than in healthy control subjects. This indicates that the eNOS 4a allele may be one of the risk factors for ESRD in ESRD patients. Another study by Bellini *et al*., (2007) also demonstrated strong correlation between eNOS 4a polymorphism and ESRD risk while Marson *et al*., (2011) reported contrary finding. VNTR in Intron4 of eNOS gene have been reported to be associated with reduced eNOS expression and enzymatic activity (Zhu *et al*., 2014). Tsukada, in their findings reported that homozygous 4bb was associated with high concentration of NO while homozygous 4aa exhibited lowest NO levels (Tsukada *et al*., 1998). Our study also observed frequencies of variant genotypes in rs2070744 polymorphism. The study observed high frequency of heterozygous TC genotype in CKD patients than in control group and the difference was found to be statistically significant(p=0.005). Genotype TT was observed with high frequency in controls than in CKD patients (71.43% vs 48.57%). This observation was consistent with reports of (Asakimori *et al*., 2004; Ahluwalia *et al*., 2008) who reported that T786C was associated ESRD risk. The T786C polymorphism located in the promoter region of eNOS gene occur when Thymidine is replaced with Cytosine. Such alteration may cause a reduced promoter activity which will lead to compromised production of eNOS enzyme and thus decreased NO production. This increases the risk of development of CKD and other diseases. Asakimori reported significantly higher frequency of both homozygous genotype CC or heterozygous TC genotype in CKD patients than in healthy controls (Asakimori *et al*., 2004). This variant may be a risk factor for development of CKD as observed in our study. This may be because C allele mutation at the T786C locus was reported to cause systemic or intraglomerular hypertension and increased glomerular pressure, which is an essential factor for glomerulosclerosis. Although, this study also observed that the homozygous CC genotype occurred with equal frequency in both groups the difference between the two groups is statistically significant(p=0.005). Although there is paucity of literature on assessment of the relationship between genotype of eNOS gene and urinary concentration of heavy metal in CKD patients, this study demonstrated significant(p=0.023) difference in mean urinary Arsenic concentration in CKD patients with respect to VNTR genotypes(4b4b,4a4b,4a4a) as compared to the control subjects. However, this study also demonstrated that there was no significant(p>0.05) among the genotypes of eNOS gene with respect to other metals (UCd, UHg,

UPb). This finding is in disagreement with report of Qusay *et al*., 2022 who reported significant difference among genotypes of rs1799983(G894) in eNOS gene as related to blood cadmium in hypertensive welding laborers than in apparently healthy subjects. This study therefore demonstrated that CKD patients of the gene polymorphism VNTR may be more susceptible to CKD development secondary to heavy metal Arsenic exposure than those with the other genotypes. This is for the fact that gene polymorphism is a risk factor for human vulnerability to heavy metal toxicity and thus organ damage such as renal injury due to chronic exposure (Slamet and Sri, 2018). Gene polymorphism is therefore, a significant risk factor influencing susceptibility to heavy metal injury in human body (Qusay *et al*., 2022). The result of this study therefore indicates that heavy metals such as Arsenate can cause mutation resulting in polymorphism in the eNOS gene, resulting in a reduction in nitric oxide (NO) production.

CONCLUSION

The study found significant associations between eNOS gene polymorphisms (e.g., G894T SNP, VNTR in Intron 4 and T786C promoter region variant) and CKD susceptibility and progression to ESRD. The observed genotypic differences between CKD patients and control subjects support the potential role of genetic markers in CKD risk assessment.

The findings of this study may guide clinical interventions aimed at slowing CKD progression, including the management of genetic risk factors.

Recommendations

We recommend further studies involving the mechanism of eNOS polymorphism and its interaction with environmental factors (such as heavy metals) as risk factors of CKD.

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