



RESEARCH ARTICLE

ENDOTHELIAL NITRIC OXIDE SYNTHASE 4A/B POLYMORPHISM AND ITS INTERACTION WITH G894T VARIANTS IN TYPE 2 DIABETES MELLITUS: PREDICTING THE RISK OF DIABETIC NEPHROPATHY

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ABSTRACT

Background: Nitric oxide (NO), a vasodilator molecule, is produced from L-arginine by endothelial nitric oxide synthase (eNOS) and it regulates endothelial function. The presence of eNOS variants might further complicate endothelial dysfunction and nephropathy through reduced production of NO. This study aimed to find out the possible association and synergistic effect of eNOS 4a/b and G894T polymorphisms on the risk of diabetic nephropathy.

Patients and Methods: This study conducted on 45 patients with diabetes mellitus type 2 and 15 healthy subjects serving as a control group. All candidates were genotyped for the eNOS polymorphism using PCR-RFLP technique.

Results: there are significant differences in genotype and allele frequencies between patients and control for eNOS G894T and (4a/b) Polymorphisms with p value <0.05, and It also showed significant differences between patients with DN and patients without DN for the GT genotype, a and T alleles with p value <0.05. The subjects with GT or aa genotypes were associated with risk to develop micro and macroalbuminuria when compared with controls but the presence of both them had no significant effect.

Conclusion: Analyzing both eNOS 4a and 894T alleles revealed that in the presence of either eNOS 4a or 894T allele, the risk of developing DN increased significantly. However, the presence of both alleles together was not associated with increased risk of microalbuminuria and macroalbuminuria.

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INTRODUCTION

Diabetic microvascular complications are associated with long-term hyperglycemia in patients with diabetes. Diabetic nephropathy is a chronic complication of DM. It results from changes in blood flow in the small vessels of the glomerular capsule, the functional unit of the kidney (Eknoyan et al., 2003). The pathogenic changes of diabetic nephropathy involve several complex chains of reactions that participate in damaging and oxidative remodeling of the vessel walls of the glomerular capsule. The subsequent decreased oxygenated blood flow, loss of vasodilatory tone, and impairment of vascular wall integrity lead to irreversible damage to glomerular capillaries and, to loss of kidney function (Vujičić et al., 2012). Microalbuminuria defined as urinary albumin

excretion of 30-300 mg/24 hrs or 30 mg/g creatinine on a random sample (George et al., 2014), is an early marker of diabetic nephropathy in type 2 diabetes. Nitric oxide (NO) is produced by the action of endothelial nitric oxide synthase (eNOS) on L-arginine. Vascular endothelial nitric oxide (NO) regulates endothelial function and precipitates vasodilatory effects in multiple organs, including the kidney. There are three isoforms of NOS: endothelial NOS (eNOS), neuronal NOS (n NOS), and inducible NOS (i NOS) (Noiri et al., 2002). Each isoform is coded by separate genes with a different pattern of expression. eNOS 4a/b and G894T are two polymorphisms that are associated with a decreased eNOS activity and a reduced plasma level of NO (Zanchi et al., 2000). A variable number of tandem repeats (VNTR) in intron 4 of eNOS (NOS3) have been reported in association with cardiovascular and renal diseases (He et al., 2011). The G894T polymorphism of eNOS results in a substitution of aspartate for glutamate at amino acid position 298 of the NOS3 protein (Thameem et al., 2008). Our objective in the present study

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was to investigate the possible association and synergistic effect of eNOS 4a/b and G894T polymorphisms on the risk of type 2 diabetes and diabetic nephropathy.

PATIENTS AND METHODS

This case-control study was conducted at the Clinical and chemical Pathology and the Internal Medicine Departments of Benha University Hospitals from April 2015 to July 2016. Forty five patients (19 males and 26 females) with diabetes mellitus type 2 who were diagnosed according to the criteria established by the American Diabetes Association Expert Committee (confirmed fasting blood glucose ≥ 126 mg/dl (7 mmol/l) and/or 2-h postprandial glucose level ≥ 200 mg/dl (11.1 mmol/l) on more than one occasion) (Expert Committee on the Diagnosis and Classification of Diabetes Mellitus (2003)), and 15 healthy age and sex matched subjects serving as a control group were enrolled in the study.

Inclusion criteria: were age at diagnosis of diabetes ≥ 30 years and the duration of diabetes ≥ 5 years, while **Exclusion criteria** were type 1 diabetes mellitus, type 2 diabetes mellitus patients of less than 5 years duration and other causes of nephropathy were excluded. Patients were further classified according to their albumin / creatinine ratio (ACR) into three groups:

Group I: 15 patients with type 2 diabetes and non-apparent diabetic nephropathy (Normoalbuminuric diabetic group). The albumin/creatinine ratio in these patients was less than 30 mg/g creatinine.

Group II: 15 patients with type 2 diabetes (microalbuminuric diabetic group) The albumin/creatinine ratio in these patients was 30 – 300 mg/g creatinine.

Group III: 15 patients with type 2 diabetes and proliferative diabetic (Macroalbuminuric diabetic group). The albumin/creatinine ratio of these patients was more than 300 mg/g creatinine. All patients were subjected to full history taking included questions about smoking habits, history of hypertension and type 2 diabetes, and current medication used and clinical examination. For all participants the following investigations were done FBG, HbA1c, total cholesterol, TG, HDL-C, LDL-C, creatinine. All candidates were genotyped for the eNOS polymorphism using PCR-RFLP technique.

Ethical consideration: The study was performed according to the principles approved by the local ethics committee and written informed consent from every subject in the study was taken prior to be involved in the study.

Methods

FBG, total cholesterol, TG, HDL-C, creatinine were performed on (Biosystem A15 automated analyser) according to manufacture instructions.

Determination of glycohemoglobin was done in whole blood using Ion Exchange Resin method.

Principle: Glycosylated hemoglobin has been defined operationally as the fast fraction hemoglobins HbA1 (Hb A1a, A1b, A1c) which elute first during column chromatography as it employs a weak binding cation exchange than non-glycosylated hemoglobin. The ratio of the absorbance of

HbA1c and total Hb of the control and test is used to calculate the percent HbA1c of the sample by using spectrophotometry at 415 nm (Nathan *et al.*, 1984).

Calculation of the albumin /creatinine ratio (ACR) in mg/g: The second morning urine samples were collected in sterile urine containers. Ten ml were separated for immediate estimation of urine creatinine and urine albumin. Urine albumin was measured by turbidimetry (Nathan *et al.*, 1984). The test was done using (BioSystem A15 spectrophotometer). ACR was expressed as (mg albumin/g creatinine) (Fox *et al.*, 2004).

Determination of of eNOS 4a/b and G894T variants genotype: Genomic DNA was extracted from EDTA blood leucocytes obtained from each patient using Gene JET Whole Blood Genomic DNA Purification Mini Kit according to the manufacture instructions (*ZYMO ESARCH*). ENOS 4a/b and G894T polymorphism identified by the polymerase chain reaction- restriction fragment length polymorphism (PCR-RFLP). The reaction mixture contains the following: 25 μ l of Dream Taq Green PCR master Mix (2X), 2.5 μ l of each primer, 5 μ l of genomic DNA, nuclease free water to a final volume 50 μ l. For detection of eNOS G894T polymorphism (*Biosearch technologies, USA*) The following primers were used for amplification: forward 5'- AAG GCA GGA GAC AGT GGA TGG A -3' and reverse 5'- CCC AGT CAA TCC CTT TGG TGC TCA -3', and for detection of 4a/b polymorphism the following primers were used for amplification: forward 5'- AGG CCC TAT GGT AGT GCCTTT -3'. and reverse 5'- TCT CTT AGTGCT GTG GTC AC -3' (Rahimi *et al.*, 2013). The DNA was amplified by initial denaturation at 95°C for 3 minutes then cycling of denaturation at 95°C for 30 second, annealing at 53°C for 30 second, and extension at 72°C for 1 minute. After 40 cycles, the reaction was extended for an additional 15 minutes at 72°C. PCR reactions were performed with PicoReal instrument. The amplified PCR product was subsequently digested at 37°C for 10 minutes with MboI (*Thermo Scientific, EU*). Agarose gel electrophoresis (3%) of the digested products was performed to determine the presence of the various genotypes: GG genotypes had band sizes of 248 bp and GT 248 bp/158 bp/90 bp, a allele a fragment with 393 bp is produced while the eNOS b allele produced a fragment with 420 bp (13).

Statistical analysis: All statistical tests were performed with SPSS version 16 soft ware (Spss Inc, Chicago, ILL Company). Categorical data were presented as numbers and percentages while quantitative data were expressed as mean and standard deviation (SD). Chi square test (X²) or Fisher's exact test (Monte Carlo method) were used, Odds Ratios (ORs) and the corresponding 95% confidence interval (CI) were calculated when applicable. Student's T-test used to compare mean of two groups of quantitative data. ANOVA test was used as tests of significance. Significant logistic regression analysis was used to detect the significant predictors of diabetic nephropathy. All p values < 0.05 were considered statistically significant. We tested the genotype frequencies for deviation from Hardy Weinberg equilibrium (HWE) proportions by using HWE calculator (Brink, 2010).

RESULTS

As regarding laboratory variables there was statistically significant increase in the mean FBS, HbA1c, Total cholesterol, triglyceride, LDL-C, and U.alb/creat ratio levels in diabetic patients compared to the control subjects ($P < 0.05$).

Table 1. The frequency of eNOS genotype and alleles in patient and control groups

		patient group (45)	Control group (15)	FET	P*	OR (95%CI)
a/b genotype n(%)	aa			$X^2=7.33$	0.007**	
	bb	24(53.3%)	2(13.3%)			7.43 (1.5-36.79)
Allels	a	48(53.3%)	4(13.3%)	$X^2=14.66$	0.001**	
	B	42(46.7%)	26(86.7%)			7.43 (1.5-36.79)
G/T genotype n(%)	GT			6.77	0.009**	
	GG	18(40.0%)	0(0.0%)			1.56 (1.24-1.95)
Allels	G	27(60.0)	15(100%)	5.58	0.018*	
	T	72(80.0%)	30(100%)			1.42 (1.25-1.61)
		18(20.0%)	0(0.0%)			

Table 2. Comparison between diabetic groups as regarding eNOS genotypes and alleles

	Group I (Normo)	Group II (Micro)	Group III (Macro)	F test	P*
a/b genotype				$X^2=4.82$	0.09
aa	5 (33.3%)	11 (73.3%)	8(53.3%)		
bb	10 (66.7%)	4 (26.7%)	7(46.7%)		
Allels n(%)	a	22 (73.3%)	16(53.3%)	9.64	0.008**
	b	8 (26.7%)	14(46.7%)		
Polymorphism n%				FET=	0.005**
GT	1 (6.7%)	8 (53.3%)	9 (60.0%)	10.56	
GG	14(93.3%)	7 (46.7%)	6 (40.0%)		
Allels				FET=	0.001**
G	29 (96.7%)	22 (73.3%)	21 (70.0%)	16.99	
T	1(3.3%)	8(26.7%)	9(30.0%)		

Table 3. The frequency of eNOS genotypes and alleles in the patient group II+III (with DN) and group I (without DN)

	Group II&III (with DN)	Group I (Without DN)	Test	P*	OR(95%CI)
Polymorphism n(%)			$X^2=3.62$	0.057	3.46 (0.936-12.74)
aa	19(63.3%)	5(33.3%)			
Bb	11(36.7%)	10(66.7%)			
Allels n(%)	a	10(33.3%)	$X^2=7.23$	0.007**	3.46 (1.37-8.69)
	b	20(66.7%)			
Polymorphism n(%)			$X^2=$	0.001**	18.31 (2.13-157.7)
GT	17(56.7%)	1(6.7%)	10.42		
GG	13(43.3%)	14(93.3%)			
Allels n(%)	G	29(96.75%)	$X^2=$	0.005**	0.087 (0.011-0.69)
	T	1(3.3%)	7.81		

Table 4. Interaction between 4a/b and G894T polymorphism

	a/b GT	aa (24) n(%)	bb (21) n(%)	FET	OR (95%CI)	P*
Total cases (45)	GT	10(41.7%)	8(38.1%)	$X^2=0.06$	1.16 (0.35-3.84)	0.807
	GG	14(58.3%)	13(61.9%)			
Group I	GT	0(0.0%)	1(10.0%)	0.536	0.0	0.464
	GG	5(100%)	9(90.0%)			
Group II	GT	6(54.5%)	2(50.0%)	0.024	1.2 (0.121-11.87)	0.876
	GG	5(45.5%)	2(50.0%)			
Group III	GT	4(50.0%)	5(71.4%)	0.814	0.4 (0.047-3.42)	0.398
	GG	4(50.0%)	2(28.6%)			

Table 5. Logistic regression showing predictors of getting nephropathy in diabetic patient

Variable	Odds Ratio (Exp B)	95% CI	P
ab (b is ref)	1.19	0.36-29.95	0.292
GT (G is ref)	9.15	3.52-2527	0.023
HbA1c	-5.28	0.0-0.379	0.016
HDL	-0.335	0.503-1.02	0.062
Creatinine	8.91	2.10-2599	0.032

While HDL-C, S.creat, and U.creat did not show statistically significant change ($P>0.05$). As regarding genotype frequencies, The GT genotype and T allele were significantly more frequent in the patient group 18(40.0%) than in the control group 0(0.0%) ($P<0.05$), The a allele and aa genotype were significantly more frequent in the patient group than in

the control group 48(53.3%) vs. 4(13.3%), and 24 (53.3%) vs 2 (13.3%), ($P<0.05$) respectively (Table1). On comparison between diabetic groups, there was statistically significant difference in the frequency of GT genotype, T and a alleles between them ($P<0.05$) (Table 2).

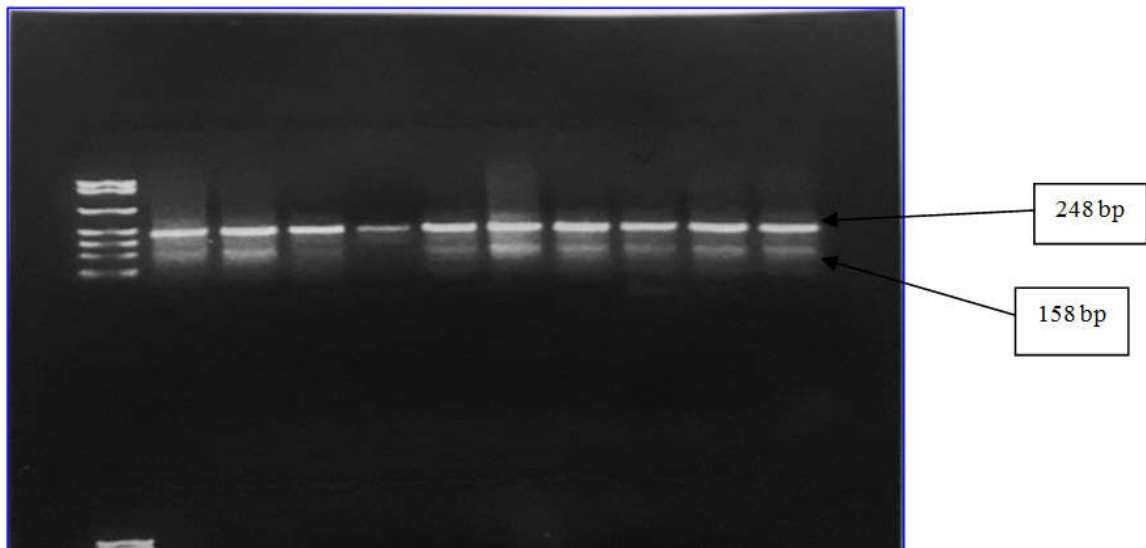


Fig.1. Bands of amplification products of G and T allele in 1% agarose gel using UV trans-illumination
1) Lane 1,3,4,5, Homozygous (GG) with one bands at 248,
2) Lane 2,6,7,8,9,10 Heterozygous (GT) with three bands at 248,158 and 90 bp

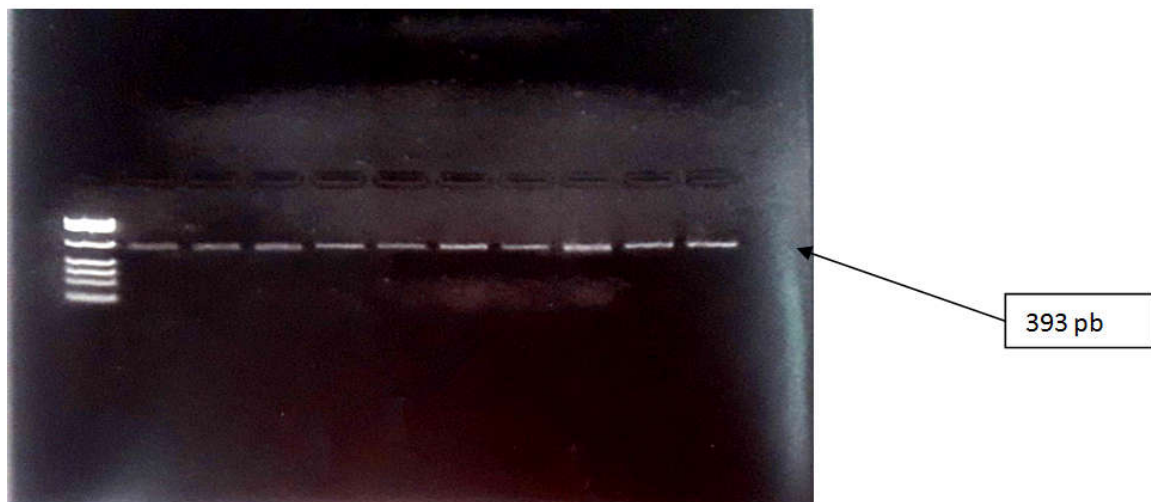


Fig.2. Bands of amplification products of a allele in 1% agarose gel using UV trans-illumination
Lane (1-10) shows (a) allele produced a fragment with 393 bp

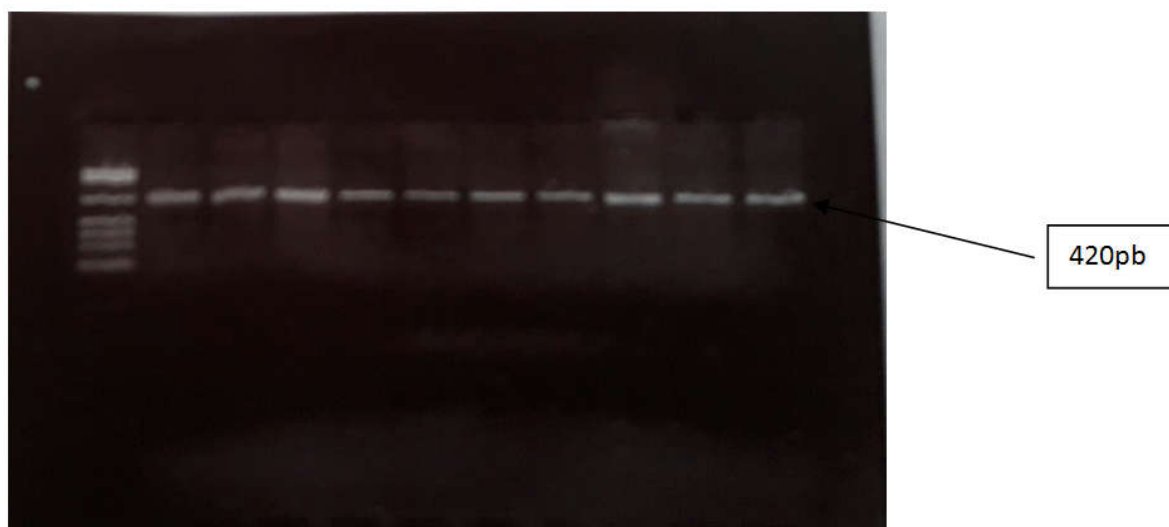


Fig.3. Bands of amplification products of b allele in 1% agarose gel using UV trans-illumination Lane 1-10
Homozygous (b/b) with one bands at 420

On comparison between each diabetic group and control group, There was no statistically significant difference in the frequency of e NOS genotypes and alleles between group I and control ($P>0.05$). However, there was statistically significant difference in frequency of a and GT genotypes in microalbuminuric group compared with control group ($P<0.05$). The subjects with the aa genotype in group II were associated with risk to develop microalbuminuria by 17.88 folds than control group, OR, 17.88, (95% CI) (2.73-116.8), Also The subjects with the GT genotype were 3.14 folds risky to develop microalbuminuria than control group OR, 3.14, (95%CI) (1.7-5.79). As regarding macroalbuminuric group, There was statistically significant difference in frequency of aa and GT genotype when compared with control group ($P<0.05$). The subjects with the aa genotype in group III were associated with risk to develop macroalbuminuria by 7.43 folds OR (7.43) 95%CI (1.23-45.01), however the subjects with the GT genotype were 3.5 folds risky to develop macroalbuminuria than control group OR(3.5), 95%CI (1.78-6.88). On comparing genotype frequency in DN patients (group II+ group III) with group I (without DN), There was statistically significant difference in frequency of GT genotype, T and a alleles in DN group compared with group I ($P<0.05$). The subjects with the aa genotype in DN group were associated with risk to develop DN by 3.46 folds than group I. The subjects with the GT genotype were 18.3 folds risky to develop DN than group I ($P<0.05$). There wasn't any statistically significant association between presence of both polymorphisms or both alleles and increasing risk of developing micro and macroalbuminuria (DN) ($P>0.05$) (Table 3) A multiple stepwise regression analysis was done using DN as a dependent factor and genotype (AA), allele A, genotype (GT), allele T, HbA1c $>7\%$, Creatinine and HDL-c as independent factors. The genotype (GT), allele T, HbA1c, serum Creatinine were found to be independent predictors for DN ($P<0.05$). (Table 5)

DISCUSSION

Diabetic nephropathy (DN) is a progressive disease elicited by chronic exposure to hyperglycemia and is a leading cause of ESRD. 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), Diabetic nephropathy, or kidney damage due to diabetes, results from changes in blood flow in the small vessels of the glomerular capsule, the functional unit of the kidney (Bennett and Aditya, 2015). Microalbuminuria is a predictive factor for cardiovascular events and nephropathy in type II diabetics (De-Zeeuw *et al.*, 2008). Endothelial nitric oxide synthase (eNOS) gene has been considered a potential candidate gene to diabetic nephropathy (DN) susceptibility. Since 1998, several polymorphisms of the eNOS gene and their association with various diseases have been identified. Some of these polymorphisms are associated with reduction of either eNOS activity or plasma concentrations. The polymorphism which potentially associated with DN is 4a/b and G894T missense mutation in exon 7 (rs1799983) (Dellamea *et al.*, 2014). The present study showed significant differences in genotype and allele frequencies between patients and control for G894T polymorphism, 18(40%) in patient group and 0(0%) in control group was GT genotype, while T allele was 18(20%) in patient group and 0(0%) in control group with p value <0.05 , (Table 1). These results coincided with many authors (Noiri *et al.*, 2002; Sun *et al.*, 2004; Ezzidi *et al.*, 2008; Ahluwalia *et al.*, 2008), who recorded a significant increase of the mutant T

allele of G894T polymorphism among cases of types 1 and 2 diabetes with nephropathy compared to control and concluded that T allele may be considered genetic risk factors for DN. In the contrary the results of Zanchi *et al.* (2000) and other studies (Badawy *et al.*, 2011; Mackawy *et al.*, 2014), revealed no significant differences in the frequencies of genotypes and alleles of G894T polymorphism between the studied diabetic patients with or without nephropathy when compared with control ($p>0.05$). This study showed significant differences between patients with DN and patients without DN regarding the genotype and allele distributions. For the 894G $>$ T SNP, the GT genotype was significantly more frequent in diabetics with DN (micro and macroalbuminuria) 17 (56.7%) than in normoalbuminuric diabetics 1 (6.7%), $p = 0.001$. and associated with risk of developing DN by 18.3folds. Also subjects with GT genotype were 3.14 folds risky to develop microalbuminuria and 3.5 folds risky to develop macroalbuminuria than control group. Similarly, the T allele was more frequent in the DN group 17(28.3%) than in diabetics without nephropathy 1(3.3%), ($p = 0.005$). These results were in agreement with Samant *et al.* (2016) whose results showed that T allele of eNOS 894G $>$ T polymorphism were significantly more frequent in diabetics with nephropathy than in diabetics without nephropathy, and with Ezzidi *et al.* (2008) (Badawy *et al.*, 2011) and Shoukry *et al.* (2012).

In the contrary Mackawy and his colleges (2014) showed that no significant differences of G894T genotypes could be detected between patients with (type 2 DM without nephropathy) who had T2DM for at least 10 years or more) and (patients suffered from type 2 DM with DN who had persistent proteinuria). However, The discrepancy of the results between the different studies can be attributed to the variability of the number of patients studied or to the ethnic differences regarding the distribution of this pattern of polymorphism or due to gene-environmental interactions. The present study showed significant differences in genotype and allele frequencies between patients and control for eNOS (4a/b) polymorphism, patient group aa genotype was 24(53.3%) ,while control group was 2(13.3%) with p value 0.007. There was no significant difference in the distribution of eNOS 4a/b genotypes between the three diabetic groups. The frequency of eNOS a genotype was 53.3% in macroalbuminuric patients, 73.3% in microalbuminuric and 33.3% in normoalbuminuric ones $p>0.05$. There was statistically significant difference in frequency of aa genotype in microalbuminuria group compared with control group and in macroalbuminuria group compared with control group ($P<0.05$) so subjects with aa genotype were associated with risk of developing microalbuminuria by 17.8 folds and 7.4 folds risky to develop macroalbuminuria. The frequency of the eNOS4a genotype in subjects with nephropathy (microalbuminuria and macroalbuminuria) were higher than in diabetic patients without nephropathy 19(63.3%) versus 5(33%) but this difference did not reach the significant level ($P = 0,057$). These results were in agreement with Shoukry *et al.* (2012) whose results showed no significant differences between patients with DN and diabetic patients without nephropathy in allele or genotype frequencies for (4a) polymorphism ($p > 0.05$) and with Santos *et al.* (2011) who reported that in Caucasian-Brazilian VNTR intron 4a/b was not associated with the renal complications of the disease . Also the results of Rahimi *et al.* (2013) in a study on 173 diabetic patients and 101 healthy cases in Iran found that the frequency of eNOS 4a allele tended to be higher in DN patients than in

the normoalbuminuric ones, but not significantly increasing risk of macroalbuminuria and microalbuminuria Also in On the other hand Mohseni *et al.* (2011) stated that eNOS 4a/b variants were not significantly associated with the risk of developing DN in T2DM patients from Iran. They explain this association of eNOS 4a/b polymorphism with the risk of developing T2DM and not with its complications such as DN might be attributed to the diverse effects of eNOS 4a/b variants on diabetes and the microvascular complications of diabetes. However He *et al.* (2011) in a meta-analysis who reported that eNOS 4a/b polymorphism (eNOS 4a versus 4b allele) were significantly associated with developing DN in an East-Asian population but not in the Caucasians, also Bellini *et al.* (2007) demonstrated a strong association between eNOS 4a allele and end stage renal disease (ESRD) among a multiethnic group from Brazil. They suggested that this polymorphism might be a genetic marker for susceptibility to ESRD even in multiethnic populations.

In the present study, and in agreement with Rahimi *et al.* (2013). analyzing both eNOS 4a and 894T alleles revealed that in the presence of either eNOS 4a or 894T allele, the risk of developing (macroalbuminuria and microalbuminuria) increased significantly. However, the presence of both alleles was not associated with the increased risk of macroalbuminuria and microalbuminuria Table (4), There may be several explanations for the absence of significant positive influence of the two alleles of eNOS 4a or 894T on the risk of developing nephropathy in spite of the significant effect of each allele on the risk of developing DN including the modulation of the risk of developing DN in the presence of both polymorphisms combined, reduction of sample size in the presence of both alleles and unidentified mechanisms underlying decreased risk of DN in the presence of both mutant alleles (Rahimi *et al.*, 2013) A multiple stepwise regression analysis was done using DN as a dependent factor and genotype (AA), allele A, genotype (GT), allele T, HbA1c >7%, Creatinine and HDL-c as independent factors. The genotype (GT), allele T, HbA1c, serum creatinine were found to be independent predictors for DN ($p < 0.05$).

In conclusion, This study suggests that the presence of either eNOS 4a/b or G894T polymorphisms increases the risk of diabetic nephropathy but they have no synergistic effect, and that genotype (GT) and (T) allele are independent predictors for DN, thus : their detection can be used as a novel method to detect susceptibility to DN in Egyptians with type 2 diabetes,

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