

## Endothelial nitric oxide synthase gene polymorphisms (G894T) in diabetes mellitus in Egypt

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### Abstract

**Objective:** Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes. Genetic predisposition has been implicated in DN. The eNOS protein synthesizes nitric oxide constitutively via a reaction including the conversion of L-arginine to L-citrulline, which involves the transfer of five electrons provided by nicotinamide adenine dinucleotide phosphate. The aim of this study is to evaluate the association of G894T polymorphisms of endothelial nitric oxide synthase (eNOS) gene with the development of diabetic nephropathy (DN) among Egyptian patients with type 1,2 diabetes mellitus in Egypt. **Methods:** Study subjects comprised 86 patients of type 2 diabetes with nephropathy, 23 patients of type 1 diabetes with nephropathy and 46 patients of type 2 diabetes without nephropathy. G894T genotypes were determined by SSP-PCR analysis. **Results:** Mutant T allele, GT and TT genotypes of G894T SNP had no significant frequencies in type 1,2 diabetic patients with nephropathy compared to those without nephropathy. **Conclusion:** These findings indicate that G894T polymorphism of eNOS gene may be not considered as genetic risk factors for DN among Egyptian type 1, 2 diabetic patients.

Abbreviations: T2DM: type 2 diabetes mellitus – DN: diabetic nephropathy eNOS : Endothelial nitric oxide synthase – SNP: single nucleotide polymorphism- SSP-PCR: sequence specific primer- polymerase chain reaction.

**Key words:** eNOS gene – Type 1, 2 diabetes - diabetic nephropathy.

### Introduction:

Diabetic nephropathy (DN) is one of the major microvascular complications of diabetes and one of the leading causes of death among patients with diabetes (Chiarelli *et al.*, 2009). Clinically, diabetic nephropathy is manifest as a progressive disease process that advances through characteristic stages. It begins with microalbuminuria (leakage of small amounts of albumin into the urine) and progresses to overt proteinuria. In a large proportion of these patients with diabetic nephropathy, renal function declines and continues to deteriorate until end stage renal disease (ESRD) is reached, and replacement therapy is indicated (Perkins *et al.*, 2003; Brenner *et al.*, 2004 and Perkins *et al.*, 2007).

The etiology of DN is multifactorial and involves both environmental and genetic factors. A

genetic predisposition, based on familial clustering of DN, has been implicated in the pathogenesis of nephropathy in type 2 diabetes mellitus (Sun J *et al.*, 2004).

Nitric oxide (NO) synthesized by endothelial nitric oxide synthase (eNOS) plays an important role in regulation of endothelial function and in the control of blood pressure.

A healthy endothelium plays a core role in cardiovascular control (Drexler H, & Horning B., 2009).

Besides its regulatory functions on vasomotor tone and blood flow, endothelial NO is known to inhibit the platelet activation and modulate migration and growth of the vascular smooth muscle (Marsden PA. *et al.* 2007).

A point mutation of guanine to thymine at nucleotide position G894T in the endothelial nitric oxide synthase (eNOS)

gene within exon 7, resulting in a replacement of glutamic acid by aspartic acid at codon 298 (Glu298Asp), has been reported to be associated with Diabetic nephropathy (Knowles RG, & Moncada S. 2004).

Several lines of evidence suggest that endothelial damage could play a key role in the development of both micro- and macroangiopathy in diabetes. (Moncada S, Higgs A. 2002).

Endothelial dysfunction plays a key role in the pathogenesis of diabetic vascular disease, including diabetic nephropathy. Endothelial-derived nitric oxide synthase (eNOS) gene polymorphisms affect eNOS activity and are associated with endothelial dysfunction. (Ahluwalia TS *et al.*, 2008). We evaluated the association of the constitutive endothelial nitric oxide synthase gene (eNOS) G897T polymorphism with types 1, 2 diabetic nephropathy.

#### **Aim of the work:**

The aim of this study is to evaluate the association of G894T polymorphism of endothelial nitric oxide synthase (eNOS) gene with the development of diabetic nephropathy (DN) among Egyptian patients with types 1, 2 diabetes mellitus in Egypt.

#### **Subjects and Methods:**

##### **Subjects:**

This study has included 109 subjects affected with diabetes type I, II. They were recruited from the Internal Medicine Specialized Hospital and Nephrology Center, Mansoura University, Egypt in the period between January and September 2009. They were divided into 2 groups in relation to the presence of nephropathy which is defined as urinary albumin excretion rate (AER) >30 mg per day. The first group consists of 102 subjects affected with diabetes type II associated with nephropathy. Their mean  $\pm$  SD age was  $58.7 \pm 6.43$  ranges from 45 to 75 years. They were in the form of 54 (52.9%) males

and 48 (47.1%) females. Of them 32 (31.4%) were affected with microalbuminuria (AER = 30-300 mg/day), while the others 70 (68.6%) showed macroalbuminuria (>300 mg/day). The other group was affected with diabetes type II without nephropathy (n=100), their mean  $\pm$  SD age was  $52.1900 \pm 8.3190$  range from 34 to 75 years, their gender was in the form of 30 (30.0%) males and 70 (70.0%) females.

##### **Method:**

##### **DNA extraction and purification**

After obtaining informed consent from all cases and controls, venous blood samples (3 ml) were collected on EDTA (ethylenediamine tetra acetate) containing tubes, DNA was extracted promptly using DNA extraction and purification kit (Gentra Systems, USA) according to manufacturer's instructions and then stored at -20 °C till use.

##### **PCR amplification**

A set of primers was designed to amplify a 206 bp fragment including the missense Glu298Asp variant (G894T polymorphism) [5'-CAT GAG GCT CAG CCC CAG-3' (forward) and 5'-AGT CAA TCC CTT TGG TGC TCA C-3' (reverse)]. The PCR fragment digested overnight at 37°C with the *Mbo*I restriction enzyme was separated by electrophoresis on 2% agarose gel and visualized by ethidium bromide staining. In the presence of a T nucleotide at position 894 corresponding to Asp 298, the 206 bp PCR product was cleaved into two fragments of 119 and 87 bp; whereas the presence of a G removed the restriction site recovering a single fragment of 206 bp.

##### **Detection of amplified and digested products**

The entire reaction volume plus 5  $\mu$ l of bromophenol blue track dye were loaded into 2% agarose gel (Boehringer Mannheim) containing ethidium bromide. Gels were electrophoresed for 30 minutes at 100 V, photographed under UV light (320 nm) and then scored for the presence or absence of an allele specific band.

**Statistical analysis:** Data were processed and analyzed using the Statistical Package of Social Science (SPSS, version 10.0). The frequency of studied allelic polymorphisms were

compared between groups by using Fisher's exact test (modified Chi square test) and. A value of  $P < 0.05$  was considered to be significant.

## Results

*Table (1): Descriptive data of studied cases of types 1, 2 diabetes mellitus with nephropathy.*

	Cases
<b>Number</b>	109
Type 1	23
Type 2	86
<b>Age (Years)</b>	
Mean $\pm$ SD	55.75 $\pm$ 9.40
Median	56.00
Range	26.00-75.00
<b>Sex (M/F)</b>	(59/50)
<b>Weight (Kg)</b>	87.86 $\pm$ 12.77
<b>Duration of diabetes (years)</b>	16.89 $\pm$ 5.94
<b>Family history (positive/negative)</b>	85/24
<b>Consanguinity (positive/negative)</b>	20/89
<b>Glycosylated hemoglobin (HbA1c, %)</b>	6.10 $\pm$ 1.59
<b>Creatinine (Cr, mg/dl)</b>	3.31 $\pm$ 2.05
<b>Cholesterol (mg/dl)</b>	212.36 $\pm$ 65.59
<b>Triglyceride (TG, mg/dl)</b>	199.20 $\pm$ 88.84
<b>High density lipoprotein (HDL, mg/dl)</b>	140.98 $\pm$ 58.78
<b>Low density lipoprotein (LDL, mg/dl)</b>	36.92 $\pm$ 12.03
<b>Microalbuminurea/ Macroalbuminurea</b>	31/78
<b>Blood pressure</b>	
Mild hypertension	40(36.7%)
Moderate hypertension	50(45.9%)
Severe hypertension	19(17.4%)
<b>Diabetic retinopathy</b>	76(69.7%)
<b>Diabetic neuropathy</b>	100(91.7%)
<b>Ischemic heart disease</b>	50(45.9%)

**Table (2):** Comparison between all cases with type 1, 2 diabetes with nephropathy and controls regarding their allele frequency and genotype distribution of G894T polymorphisms of eNOS gene.

G894T	Genotypes			Alleles	
	GG n(%)	GT n(%)	TT n(%)	G n(%)	T n(%)
All cases (n=109)	70(64.2)	24(22.0)	15(13.8)	164(75.2)	54(29.5)
H. controls (n=46)	35(67.1)	7(15.2)	4(8.7)	447(71.9)	175(28.1)
P	0.18	<b>0.38</b>	<b>0.43</b>	0.13	0.13

n= number of cases

(%) = percentage of cases

**Table (3):** Comparison between type 1 diabetes with nephropathy and controls regarding their allele frequency and genotype distribution of G894T polymorphisms of eNOS gene.

G894T	Genotypes			Alleles	
	GG n(%)	GT n(%)	TT n(%)	G n(%)	T n(%)
Type 1 (n=23)	16(69.6)	4(17.4)	3(13.0)	36(75.2)	10(29.5)
controls (n=46)	35(67.1)	7(15.2)	4(8.7)	77(71.9)	15(28.1)
P	0.57	<b>1.00</b>	<b>0.67</b>	0.48	0.48

n= number of cases

(%) = percentage of cases

**Table (4):** Comparison between type 2 diabetes with nephropathy and controls regarding their allele frequency and genotype distribution of G894T polymorphisms of eNOS gene.

G894T	Genotypes			Alleles	
	GG n(%)	GT n(%)	TT n(%)	G n(%)	T n(%)
Type 2 (n=86)	54(62.8)	20(23.3)	12(14.0)	128(75.2)	44(29.5)
controls (n=46)	35(67.1)	7(15.2)	4(8.7)	77(71.9)	15(28.1)
P	0.17	<b>0.36</b>	<b>0.57</b>	0.09	0.09

n= number of cases

(%) = percentage of cases



**Table (5):** Comparison between type 1 diabetes with nephropathy and type 2 diabetes with nephropathy regarding their allele frequency and genotype distribution of G894T polymorphisms of eNOS gene.

G894T	Genotypes			Alleles	
	GG n(%)	GT n(%)	TT n(%)	G n(%)	T n(%)
Type 1 (n=23)	16(69.6)	4(17.4)	3(13.0)	36(75.2)	10(29.5)
Type 2 (n=86)	54(62.8)	20(23.3)	12(14.0)	128(71.9)	44(28.1)
P	0.62	<b>0.77</b>	<b>1.00</b>	0.7	0.7

n= number of cases  
(%) = percentage of cases

**Table (6):** Comparison between type 1 diabetes with nephropathy ,type 2 diabetes with nephropathy and controls regarding their allele frequency and genotype distribution of G894T polymorphisms of eNOS gene.

G894T	Genotypes			Alleles	
	GG n(%)	GT n(%)	TT n(%)	G n(%)	T n(%)
Type 1 (n=23)	16(69.6)	4(17.4)	3(13.0)	36(75.2)	10(29.5)
Type 2 (n=86)	54(62.8)	20(23.3)	12(14.0)	128(71.9)	44(28.1)
Controls (n=46)	35(76.1 )	7(15.2)	4(8.7)	77	15
P	0.62	<b>0.77</b>	<b>0.92</b>	0.43	0.43

n= number of cases  
(%) = percentage of cases



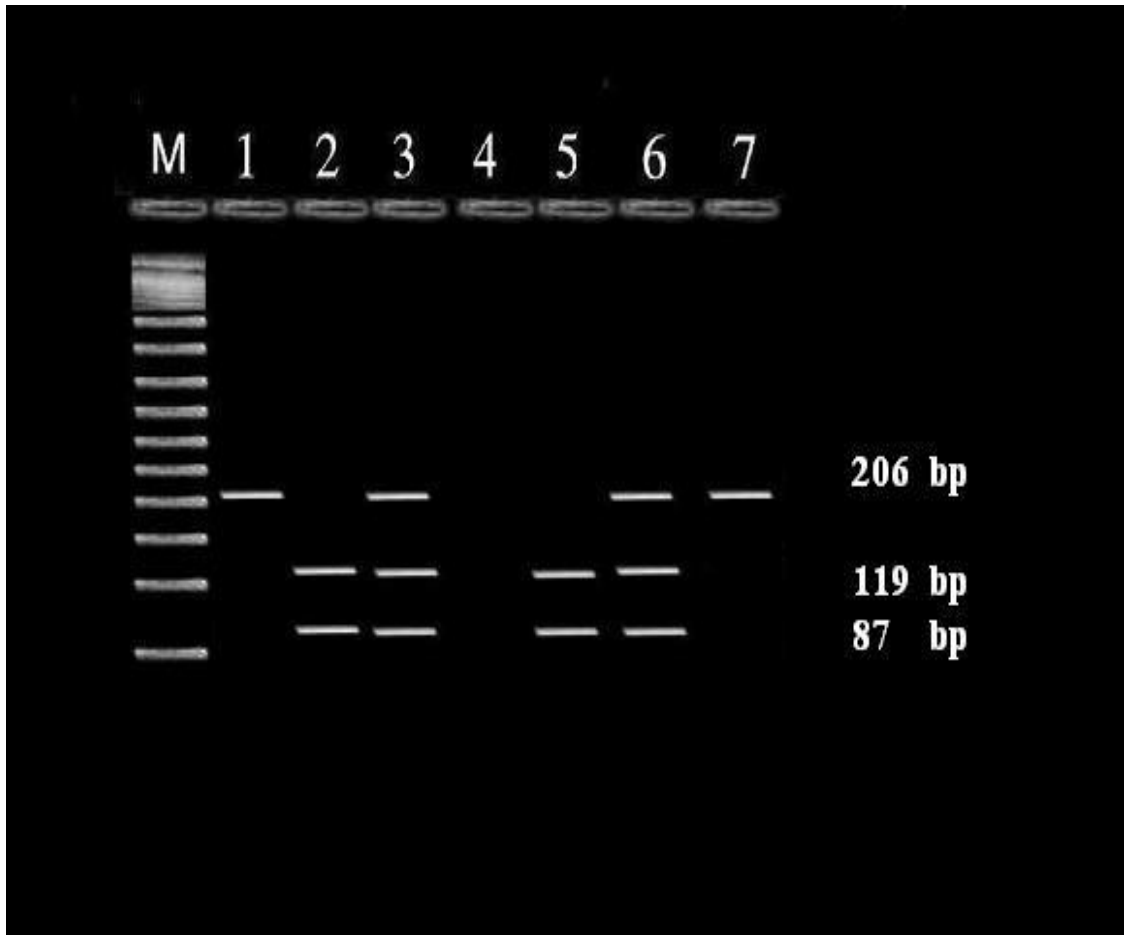


**Fig (1):** PCR amplification of G894T polymorphism of eNOS gene PCR product of G894T polymorphism have band size 206 bp. wild type GG is found which appears at 206 bp in all lanes

Lane M: DNA size marker 50 bp





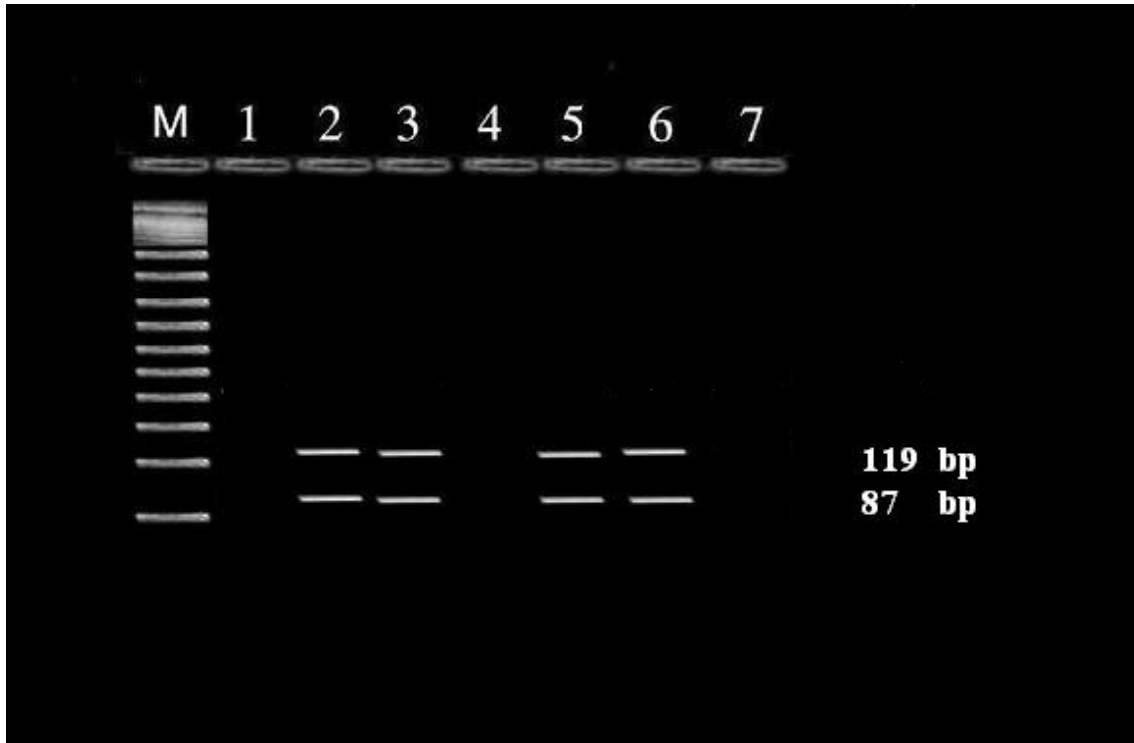


**Fig (2):** enzymatic digestion of G894T polymorphism of eNOS gene. wild type GG is found which appears at 206 bp only lanes 1 and 7

Digestion of PCR product of G894T polymorphism of eNOS gene using *MboI* enzyme. which digests the 206- bp fragment into 119- and 87- bp fragments (heterozygous mutated genotype GT which has 206, 119, 87 bp fragments lanes 3 and 6 ) (homozygous mutated genotype TT is found which has 119, 86 bp fragments lanes 2 and 5)

Lane M: DNA size marker 50 bp





**Fig (3):** enzymatic digestion of G894T polymorphism of eNOS gene. Digestion of PCR product of G894T polymorphism of eNOS gene using *MboI* enzyme. which digests the 206- bp fragment into 119- and 87- bp fragments (homozygous mutated genotype TT is found which has 119, 86 bp fragments lanes 2 ,3,5 and 6)  
Lane M: DNA size marker 50 bp



## Discussion:

Our data showed that homozygous mutated TT genotype, mutant T allele of G894T polymorphism had not significant frequency among cases of types 1, 2 diabetes with nephropathy compared to controls. Thus TT genotype and T allele may be not considered as genetic risk factors for diabetic nephropathy.

The study of Anne Zanchiet *al.* (2007) that included 82 American type 1 diabetics with nephropathy and 85 controls diabetic patients without nephropathy showed that the frequencies of eNOS G894T gene variant genotypes and alleles in the patients with DN patients were not significant among cases ( $P=0.37$ ) **Anne Zanchiet *al.* (2007)**. In a study of a sample of American patients of type 1 diabetes mellitus with diabetic nephropathy found that The frequency of TT homogenetic type and T allele (28.21%, 53.85%) was higher in patients of type 1 diabetes mellitus with diabetic nephropathy than controls (17.54%, 31.58%) **Sun *et al.*, (2007)**. Also observed that among American population, markedly elevated mutation was observed in patients with diabetic nephropathy as compared with normal people (20.8% vs 7.3%, ). Also, Noiri *et al.* (2005)

In a study of Hua Caiet *al.* that included 574 Australian diabetic patients with nephropathy and controls 324 diabetic patients without nephropathy showed that 48 % patients with diabetic nephropathy had eNOS gene mutation, ( $P = 0.30$ ) **Hua Caiet *al.* (1998)**. The genotype distribution and allele frequencies between patients (GG=51.9%, GT= 40.6%, TT= 7.5%, G allele 72%, T allele 28%) and not different between men and women ( $P = 0.700$ ). The eNOS TT and TG genotypes were not associated with age, age at onset of documented diabetes, BMI, systolic and diastolic blood pressures (BPs), lipid profile plasma creatinine and glycosylated hemoglobin (HbA1c) levels, or urinary

albumin index (UAI: albumin/creatinine ratio).

They reported that homozygous mutated TT genotype, mutant T allele of G894T polymorphism had significant frequency among cases of types 1, 2 diabetes with nephropathy compared to controls. Thus TT genotype and T allele may be considered as genetic risk factors for diabetic nephropathy.

The study of Tarunveer Singhet *al.* that included 195 India type 2 diabetics with nephropathy and controls 255 diabetic patients without nephropathy showed that the frequencies of eNOS G894T gene variant genotypes and alleles in the patients with DN patients with nephropathy were significantly higher than those in patients without nephropathy ( $P=0.03$ )

**Tarunveer Singh *et al.* (2008)**. In a study of a sample of India patients of type 2 diabetes mellitus with diabetic nephropathy Sun and colleagues found that The frequency of TT homogenetic type and T allele (28.21%, 43.85%) was significantly higher in India patients of type 2 diabetes mellitus with diabetic nephropathy than patients without nephropathy (17.54%, 31.58%)

The genotype distribution and allele frequencies between patients (GG=38.5%, GT= 33.3%, TT= 28.2%, G allele 56.15 %, T allele 43.85%)

Our study showed that there were no significant differences in genotype distribution of G894T polymorphism of eNOS gene among types 1,2 diabetic patients with macroalbuminuria compared to those with microalbuminuria .

Interestingly, the frequency of homozygous mutated TT genotype of G894T polymorphism of eNOS gene was no significant among Cases with

gender, among types 1,2 (p= 0.31) , P=0.94 respectively

We noted that, there is no significant difference among cases of diabetes types 1, 2 with nephropathy regarding their genotypes of G894T polymorphism of eNOS gene when they classified to subgroups according to parental consanguinity, p= 0.24 , P=0.38 respectively

Our data indicate that no significant difference between positive and negative family history subgroups of types 1, 2 diabetic patients with nephropathy regarding their genotypes of G894T polymorphism.

p= 0.47 , P=0.86 respectively

Our data showed that homozygous mutated TT genotype, mutant T allele of G894T polymorphism had a significantly higher frequency in ischemic heart disease (coronary heart disease) among cases of type 1 diabetes with nephropathy compared to cases of type 2 diabetes with nephropathy (p= 0.02 , P=0.042 respectively). Thus TT genotype and T allele may be considered as genetic risk factors for coronary heart disease .

Our study indicated that there were no association between G894T polymorphism and hyperlipidemia among subgroups of patients with types

1, 2 diabetes which were classified regarding their lipid profile (cholesterol, triglycerides, HDL and LDL).

**Conclusions:** Our findings indicate that G894T polymorphisms of eNOS gene may be not considered as genetic risk factors for DN among Egyptian type 1, 2 diabetic patients.

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## التعدد الجيني لجين تخليق أكسيد النيتريك في مرضى السكر المصريين

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اعتلال الكلي السكري هو احد المضاعفات طويلة الامد الناتجة عن مرض السكري. يبدأ اعتلال الكلي السكري بتلف بسيط و تصفيه عالية خلال الكلي ويتقدم الي مرض كلوي مزمن خاصة ان لم يتم علاجه.

فالنسيج الداخلى للأوعيه الدمويه يتحكم فى الأنقباض و الأنبساط عن طريق انتاج مواد تنظم الأوعيه الدمويه وحالة التجلط ونمو الخلايا وكذلك حركة كرات الدم البيضاء . يعتبر (No) من أهم هذه المواد الى ينتجها النسيج الداخلى للأوعيه الدمويه . يلعب (No) دورا هاما فى انبساط العضلات اللاراديه المبطنه لجدار الأوعيه الدمويه وكذلك يمنع التصاق الصفائح الدمويه وخلايا الدم البيضاء بجدار الوعاء الدموى . التحورات القاعدية المفردة فى الجين المسئول عن إنتاج إنزيم eNOS ظهرت فى الحالات المرضيه لاعتلال الكلي السكري فى بعض المجموعات العرقية. يوجد واحد من التحورات القاعدية المفردة المعروفه وهو (جونين 894ثايمين).

تم التخطيط لهذا البحث لدراسة تأثير التحورات القاعدية المفردة (جونين 894 ثايمين)علي القابلية الجينية لحدوث اعتلال الكلي السكري بين مرضي المصريين بالسكري النوعان الاول و الثاني. اشتملت هذه الدراسة علي 109 حالة مصابة بالسكري النوعان الاول و الثاني المصاحب باعتلال الكلي. تم اختيار تلك الحالات من مركز الكلي والمسالك البولية. بالاضافه الي 46 حالة مصابة بالسكري النوع الثاني غير مصاحب باعتلال الكلي وتم اختيارهم من مستشفى الباطنه التخصصي. بعد استخلاص الحامض النووي DNA من الدم , تم تعيين التركيب الجيني للتحورات القاعدية المفردة(جونين 894 ثايمين) لجين eNOS باستخدام تفاعل البلمره المتسلسل والتقطيع بانزيمات التقطيع. تم تعيين نواتج التقطيع الانزيمي عن طريق وضعها فى قالب من الاجاروز تركيز 3% والذي يتم تلويينه بمادة بروميد الاثيديم باستخدام الهجرة الكهربائية لمدة 30 دقيقة عند 100 فولت



ثم تصوير القلب باستخدام الاشعة الفوق بنفسجية وتم عمل صورة بافلام البولارويد لتسجيل النواتج.

وجد أن...

لا توجد نسبة احصائية معنوية بالتركيب الجيني ( ثايمين ثايمين) الخاص بالتحور القاعدي المفرد (جونين 894 ثايمين) والتركيبات الجينية المشتركة (جونين ثايمين) فى الحالات المرضيه لاعتلال الكلي السكري

لا يوجد فروق معنوية في نسب التحور ( جونين 894 ثايمين) عند تقسيم حالات السكري النوعان الاول و الثاني المصاحب باعتلال الكلي السكري حسب النوع , زواج الاباء عن قرابه, ضغط الدم المرتفع, نسبة الالبومين في البول, التحكم في نسبة السكر في الدم, التهاب العيون, التهاب الاعصاب و حسب التاريخ العائلي للمرض .

بمقارنة الحالات حسب مرض نقص التروية القلبية وجد ان حالات السكري النوعان الاول و الثاني المصاحب باعتلال الكلي السكري لديهم نسبة احصائية معنويه .