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Allelic Polymorphism in the Endothelial Nitric Oxide Synthase Gene in Coronary Artery Diseases

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Abstract:

Nitric oxide (NO) has an important role in the relaxation of the vascular smooth muscles, inhibits adhesion of platelets and leucocytes to the endothelium, reduces vascular smooth muscle cells migration and proliferation and limits the oxidation of atherogenic LDL.

Nitric oxide is constitutively produced in the endothelium of blood vessels from L- arginine by the enzyme endothelial nitric oxide synthase (eNOS).

Polymorphism in the (eNOS) gene is an important risk factor in the pathophysiology of coronary artery disease(CAD) .

In this study the polymorphism in (eNOS) gene was investigated in 30 patients with CAD and 20 control subjects using polymerase chain reaction (PCR) analysis. Patients were classified into 10 patients with unstable angina and 20 patients with myocardial infarction

Results : The distribution of (eNOS) genotypes in patients affected by unstable angina was 50% for GG genotype , 40% for GT genotype and 10% for TT genotype and in patients affected by myocardial infarction was (45%) for GG genotype, (35%) for GT genotype and (20%) for TT genotype.

In control subjects it was (50%) for GG genotype, (45%) for GT genotype and (5%) for TT genotype.

Conclusion: there is a great controversy about the role of (eNOS) gene polymorphism in the pathophysiology of CAD.

Introduction

Coronary artery disease is a multifactorial disorder with genotype and environmental interactions having an important role in its development. **(Francisco et al, 2006)**

Nitric oxide (NO) has been recognized as a hormone with a broad range of effects. One of the major effects of NO is to induce the relaxation of smooth muscles of blood vessels, an important factor in the regulation of blood pressure, and was previously recognized as the Endothelium-Derived Relaxing Factor. **(Albrecht et al, 2003)**

Nitric oxide binds to the heme moiety at the active site of soluble guanylate cyclase initiating a confirmational change which increases the production of cyclic guanosine mono phosphate (cGMP) & facilitates protien phosphorylation by the cGMP-dependant protien kinase which lead to muscle relaxation. **(Lucas et al, 2000)**

There are three isoforms of the nitric oxide synthase enzyme: the neuronal isoform (nNOS or NOS I), the inducible isoform (iNOS or NOS II) and the endothelial

isoform (eNOS or NOS III). All have a similar molecular structure and require multiple cofactors, including flavins, heme, NADPH and tetrahydrobiopterin to maintain NO production. **(Huang, 2003)**

Neuronal and endothelial isoforms are constitutively expressed and are activated by calcium-calmodulin. The inducible isoform is regulated primarily at the transcriptional level, independent of agonist stimulation and intracellular calcium levels.

Among the reported polymorphisms of the eNOS gene, a significant association of the Glu298Asp polymorphism of the eNOS gene with coronary artery diseases has been reported.(**Leeson et al,2002**)

Aim Of Work: to assess the possible association of (eNOS) gene polymorphism in the pathogenesis of (CAD)

Subjects and Methods:

Fifty subjects were participated in the procedures of this study and were divided into:
1. Control group: twenty male subjects, showing no symptoms or signs of myocardial infarction or angina, were selected and assigned as control group.
2. Patients group: included thirty male patients, suffering from coronary artery disease.

Patients were classified into two groups:
-Unstable Angina (UA) patients: included ten patietns suffered from unstable angina pectoris .
- Myocardial Infarction (MI) patients: included twenty patients with myocardial infarction (MI).

The following was done:

1 History taking for age, hypertension, diabetes, smoking and family history of CAD.
2 Serum blood glucose, cholesterol and triglycerides.
3 DNA extraction from blood.
4 Genotyping of (eNOS) gene by PCR amplification of exon 7 using specific primer followed by restriction enzyme digestion.
5Agarose gel electrophoresis.

Methods:
Speciment collection:
- Morning 10 ml blood samples were collected from all subjects after 12 hours fasting.
- The samples were divided as two milliliters (2ml) blood on sodium fluoride for determination of glucose**,(Tribe & Poston,1986.).**
- Four milliliters (4ml) blood were left for 10 minutes to clot and then centrifuged at 3000 rpm for 5 minutes. The serum was then separated for determination of total cholesterol **(Allain et al,1974)** and triglycerides (**Buccolo & David ,1973).**
- Four milliliters (4ml) blood on EDTA were stored at – 80^{O}C to be used for geneotyping of (eNOS) gene.

Molecular biology testing:
a-DNA extraction
DNA was extracted using QIAamplification extraction kit (QIAGEN)
b- Primer sequences
The sequence of primers used for amplification of eNOS exon 7 was 5'-GACCCTGGAGATGAAGGCAGGAGA (G894T forward) and 5'-ACCACCAGGATGTTGTAGCGG-TGA (G894T reverse).
c- PCR (Amersham Pharmacia Biotech, Piscataway, NJ, USA).
d- Agarose Gel Electrophoresis

e- Restriction Enzyme Cleavage

Statistical analysis of data using T-Student Test Significance was adopted at $P < 0.05$.

Result:

No statistical significant differences in age for control & all patients ($p<0.05$) was found (table-1).
As regards the risk factors including hypertension, diabetes and smoking , there were high significant differences between control & all patients.

Table (1): Clinical data of all studied groups

Variables	Control (n=20)	All patients (n=30)
Age (years)	49.30±1.68	50.66±2.06
Risk factors Hypertension Yes No	 0 (0%) 20 (0%)	 9 (30.0%)* 21 (70.0%)
Diabetes Yes No	 0 (0%) 20 (100%)	 7 (23.3%)* 23 (76.7%)
Smoking Yes No	 7 (35%) 13 (65%)	 20 (66.7%)* 10 (33.3%)
Family history Yes No	 3 (15%) 17 (85%)	 13 (43.3%)* 17 (56.7%)

NB: * = the test is significant in comparison to control group ($p<0.05$).

Regarding the mean values (±SD) of laboratory data of studied groups there were high significant difference in blood glucose level, serum cholesterol & triglycerides between control & all patients (table-2).

Table (2): Laboratory data of studied groups

Variables	Control (n=20)	All patients (n=30)
Glucose	112.30±35.00	153.66±95.38*
Cholesterol	161.80±23.17	191.36±41.20*
Triglycerides	82.50±39.06	99.13±50.75*

The percentage of normal GG genotyping was (50%), (46.7%), (50%) and (45%) for control, all patients, unstable angina and myocardial infarction groups respectively with no statistical significant difference between the studied groups regarding that genotype.
The GT genotype (heterozygeous abnormality) genetic distribution was (45%), (36.7%), (40%) and (35%) for control, all patients, unstable angina and myocardial infarction groups respectively and also there was no statistical significant difference between the studied groups regarding that genotype.
While the percentage of TT genotype (homozygous abnormality) was (5%), (16.7%), (10%) and (20%) for control, all patients, unstable angina and myocardial infarction groups respectively. There was higher incidence of TT traits in MI group (20%) which was statistically significant in comparison to control group (5%), while there was statistically non significant difference in TT traits when comparing all patients and unstable angina groups (16.7% and 10% respectively) in comparison to control group (5%) (table-3).
The alleles distribution in all cases. The G allele represents (72.5%), (65%), (70%) and (62.5%) for control, all patients, unstable angina and myocardial infarction groups respectively. While T allele represent (27.5%), (35%), (30%) and (37.5%) for control, all patients, unstable angina and myocardial infarction groups respectively. There was statistically significant increase in T allele in MI group (37.5%) in comparison to control group (27.5%). While There was statistically non significant difference in G allele or T allele when comparing all patients group or unstable angina group with control group or with each other (table-3).

Table (3): polymorphism of eNOS in studied groups

Variables	Groups			
	Control (n=20)	All patients (n=30)	Angina (n=10)	MI (n=20)
NOS gene				
GG	10 (50%)	14 (46.7%)	5 (50%)	9 (45%)
GT	9 (45%)	11 (36.7%)	4 (40%)	7 (35%)
TT	1 (5%)	5 (16.7%)	1 (10%)	4 (20%)*
NOS Allele				
G	29 (72.5%)	39 (65%)	14 (70%)	25 (62.5%)
T	11 (27.5%)	21 (35%)	6 (30%)	15 (37.5%)*

Regarding laboratory findings, there was statistically significant increase in serum levels of glucose, cholesterol and triglycerides in TT genotype cases in comparison to cases with GT or GG genotypes; and also there was statistically significant increase in those levels in GT genotype cases in comparison to cases with GG genotype (table-4).

Table (4): Correlation between eNOS genotypes (G894T) and different laboratory findings for all patients

	GG		GT		TT		P value
	Mean	SD	Mean	SD	Mean	SD	
Glucose	102.78	10.80	149.00	98.66	316.00	25.71	<0.001**
Cholesterol	165.13	21.10	188.90	35.41	255.40	22.13	<0.001**
Triglycerides	80.13	35.67	147.54	29.99	162.80	35.51	<0.001**

Figures: (1) & (2) show the correlation between both clinical & laboratory data.

Figure (1): comparison for (eNOS) genotypes regarding clinical data in all patients.

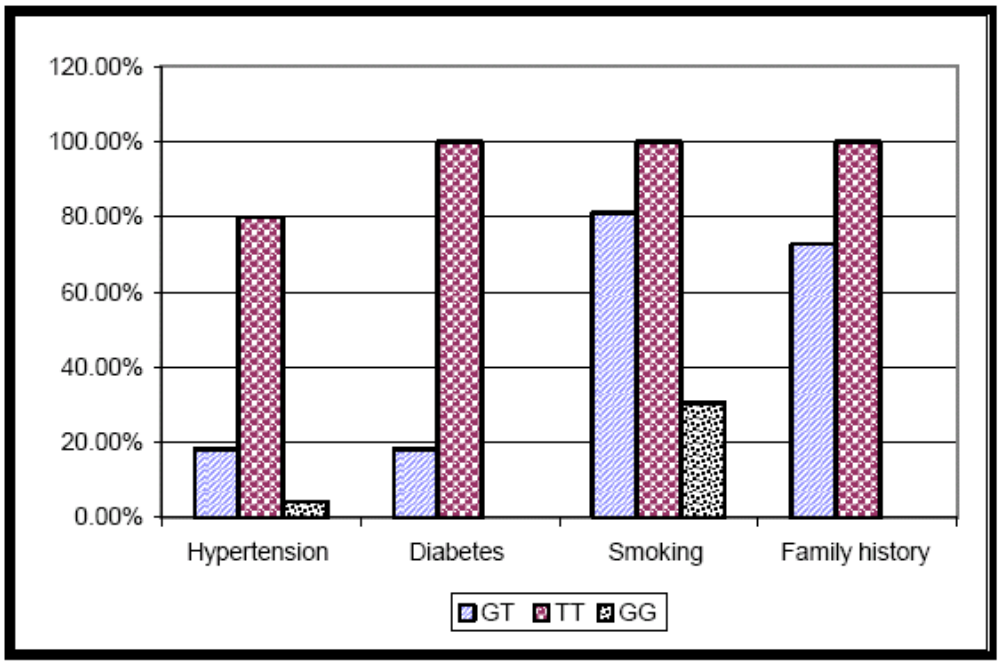
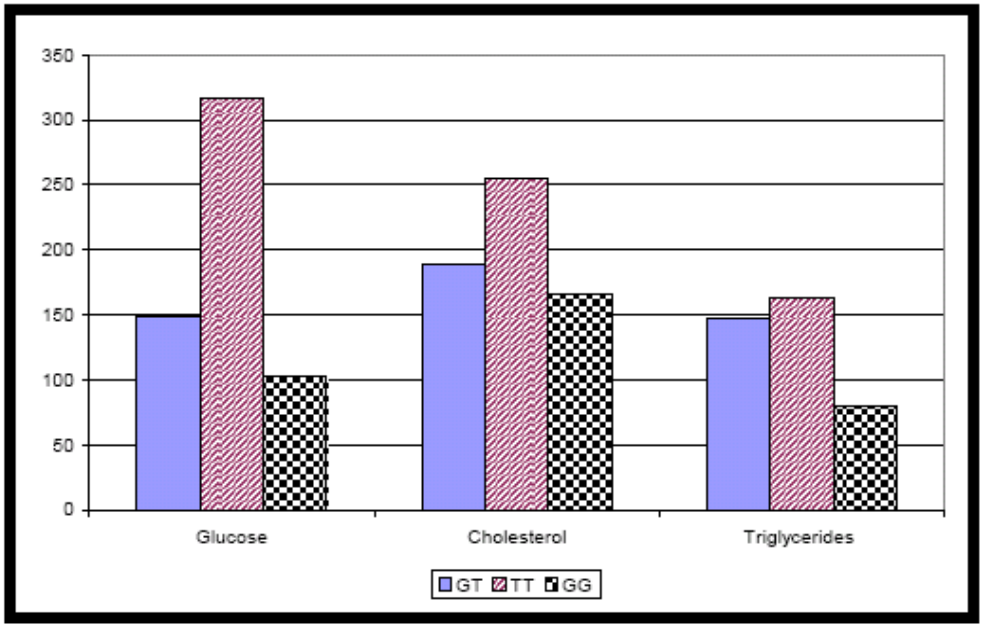


Figure (2): comparison for (eNOS) genotypes as regard laboratory findings.



Discussion:

Multiple genetic factors including mutations and polymorphisms to several genes have been associated with the risk of cardiovascular disease.

Due to the protective role of nitric oxide against important events during atherogenesis, the endothelial nitric oxide synthase gene has gained special importance in the pathogenesis of coronary heart disease **(Salimi et al, 2006).**

All the selected subjects either in the patients or control groups were matched for age, there was no statistical significant difference in the mean (±SD) age of all patients, in comparison to control group.

As regards to the risk factors as hypertension diabetes, smoking, hypercholesterolemia and increased triglycerides, there were statistical high significant difference in all patients in comparison to control group.

These results are in agreement with the results of **Antoniades et al., (2005) & Kerkeni et al., (2006),** they found that the incidence of diabetes mellitus, hypertension, hypercholesterolemia and increased triglycerides were higher in coronary artery disease group**,** in comparison to control group.

These results are in disagreement with **Colombo et al., (2003)** , **Park et al (2004) ,Cam et al., (2005) &Jaramillo et al., (2005)** , who reported that there were no significant correlation between diabetes mellitus , hypertension, hypercholesterolemia and increased triglycerides & coronary artery disease.

Among many genetic polymorphisms of the (eNOS) gene, the most studied polymorphisms were (eNOS) gene intron 4b/a VNTR (intronic polymorphism), (eNOS) Glu^{298} →Asp (exonic polymorphism) and T^{786}→C polymorphisms (promoter region polymorphism). It has become clear that the intron 4b/a, the Glu^{298} →Asp, and the T^{786}→C variants have important implications in cardiovascular diseases **(Yoshimura et al., 1998; Hingorani et al., 1999; Yoshimura et al., 2000).**

Because nitric oxide is an antiatherogenic, antiproliferative, and antithrombotic factor, the decrease in NO production and bioactivity influences vascular homeostasis. Modulation and regulation of vascular tone and vasomotion could explain why diverse pathological conditions such as hypercholesterolemia, hypertension, diabetes and cigarette smoking are all considered risk factors for atherosclerosis **(Yetik-Anacak and Catravas, 2006).**

The results of this study demonstrated that the distribution of the genotypes of the G894T of (eNOS) gene [GG (normal genotyping), GT (heterozygeous abnormality) and TT (homozygous abnormality)] were (45%), (50%), (5%), respectively, in control subjects and (50%),(40%), (10%), respectively, in unstable angina group, while in MI group it was (45%), (35%),(20%), respectively. In MI group there was statistically significant higher frequency of TT (homozygous abnormality) when compared with that of control group while there was statistically non significant difference when comparing normal GG genotyping or GT (heterozygeous abnormality) with control group.

In all patients group and also unstable angina group there was statistically non significant difference when comparing normal GG genotyping, GT (heterozygeous abnormality) and TT (homozygous abnormality) with control group.

In our work,the frequency of T allele was higher in MI group (37.5%) ,than control group (27.5%) while no statistical significant difference was found between all patients group (35%) or unstable angina group (30%) in comparison to control group. The frequency of G allele was (72.5%) in control group with no statistical significant difference detected in comparison to all patients (65%) , unstable angina group (70%) or MI group (62.5%).

These data support the hypothesis that (eNOS) gene polymorphism is important in the pathophysiology of cornary artey diseases especially in pathogenesis of myocardial infarction

In agreement with our results, **Yoshimura et al.,(1998), Hingorani et al.,(1999), Liu et al.,(2000), Lembo et al.,(2001), Fatini et al.,(2002)**

, Poulin et al., (2004), Antoniades et al.,(2005), Jaramillo et al.,(2005)

, Kerkeni et al., (2006).

In contradiction to our results **Park et al., 2004** reported non significant association in genetic distribution between acute coronary syndrome group in comparison to control subjects as regards GG, GT, TT genotypes.

In conclusion, there is a great controversy about the role of (eNOS) polymorphism in the pathpphysiology of coronary artery disease. The present study is one step on the way to explore this role. It was found –in the present study- a relation between (eNOS) polymorphism & development of MI in CAD as several previous reports documented this role although some others deny this role & whatever the situation it is recommended to apply further studies on the same topic but on a wide scale of patients.

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تعدد الاشكال الجينيه لجين الانزيم المصنع لمادة اوكسيد النيتريك داخل الخلايا الجدارية فى مرضى الشريان التاجى

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الملخص العربي

و يطلق مادة (سيتريولين-ل) الى (أرجينين-ل) مادة النيتريك أوكسيد تنتج بواسطة الانزيم المصنع للنيتريك أوكسيد و الذى يؤكسد مادة النيتريك أوكسيد

بعض التغيرات التى تحدث فى الانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية ؛ ينتج عنها قصور فى وظائف
الشريان التاجى

و قد اجريت هذه الدراسة على خمسين شخصا منهم ثلاثون شخصا مصابين بأمراض فى الشريان التاجى و عشرين شخصا
كمجموعة ضابطة بغرض اكتشاف التغير فى جين الانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية فى مرضى الشريان التاجى بالمقارنة مع أشخاص طبيعين
و قد تم تقسيم المرضى الى مجموعتين رئيسيتين
المجموعة الاولى تشمل عشرة مرضى مصابين بقصور فى الشريان التاجى
المجموعة الثانية تشمل عشرين مريضا مصابين بالذبحة الصدرية
و قد تم عمل ما يلى:
أخذ التاريخ المرضى
رسم قلب كهربائى ؛ موجات فوق صوتية للقلب
من الدم استخلاص لل- (ا.ن.د)
التميز الوراثى باستخدام تقنية ال- (ار.سى.بى)
استخدام تقنية الفصل الكهربائى بمادة ال-(أجاروز جل)

و قد اثبتت نتائج هذة الدراسة مل يلى:

للانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية لا توجد علاقة ذات دلالة احصائية بين جميع المرضى مقارنة بالمجموعة الضابطة فى التحور الجينى-GT

للانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية لا توجد علاقة ذات دلالة احصائية بين مرضى قصور الشريان التاجى مقارنة بالمجموعة الضابطة فى التحور الجينى-TT

للانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية فى مرضى الذبحة الصدرية زيادة ذات دلالة احصائية مع وجود فارق احصائى معنوى مقارنة بالمجموعة الضابطة فى التحور الجينى- TT

للانزيم المصنع لمادة النيتريك أوكسيد داخل الخلايا الجدارية فى مرضى الذبحة الصدرية زيادة ذات دلالة احصائية مع وجود فارق احصائى معنوى مقارنة بالمجموعة الضابطة فى التحور الجينى- T