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Association of a 27-Bp Repeat Polymorphism in Intron 4 of Endothelial Constitutive Nitric Oxide Synthase Gene with Coronary Artery Disease in a Tunisian Population

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Abstract

Background: Nitric oxide (NO) plays a major role in the regulation of vascular tone associations between NO genotypes. Coronary artery disease (CAD) and other risk factors have been described by many authors. The aim of this study was to investigate the role of endothelial nitric oxide synthase (eNOS) gene intron 4a/b variable number of tandem repeats (VNTR) polymorphism and other risk factors in the developement of CAD in subjects of the Tunisian population.

Method: A total of 274 Tunisian patients with CAD and 162 healthy controls were included in the study. The eNOS gene inton 4a4b variable number of tandem repeats polymorphism was analysed by PCR. The plasma lipid levels and other risk factors were also determined in all subjects.

Results: A significant differences in genotype distribution and allele frequency was observed between patients and controls. Patients with CAD had a frequency of 2.4% for the 4a4a genotype, 63.9% for the 4a4b genotype and 33.7% for the 4b4b genotype. The controls had a frequency of only 6.3% for the 4a4a genotype, 30.4% for the 4a4b genotype and 63.3% for the 4b4b genotype (2=43.75, p=0.000). The CAD patient group showed a significant higer frequency of the 4a allele compared to the controls (0.34 vs. 0.21; ^{2}=15.31, p=0.000). The odds ratio of CAD for 4a vs. 4b allele frequency was statistically significant 1.9 [1.37-2.64] at 95% CI.

The mean serum NO levels in CAD was no significant association between the patients and the control group (p=0.49).

Conclusion: The present study showed a significant and independent association between the eNOS 4a4b gene polymorphism (presence of 4a allele) and CAD but no significant difference between eNOS genotypes and NOx concentrations in CAD patients and controls in the Tunisian population.

Keywords: eNOS; Genotype; Risk factors; Coronary artery disease; eNOS gene intron 4a/b; VNTR

Introduction

Nitric Oxide (NO), a potent vasodilator functioning as an important key factor in the atherosclerotic properties of the endothelium, is generated by endoyhelium and produced from L-arginine by endothelial nitric oxide synthase (eNOS) [1]. It mediates vascular smooth muscle relaxation. It is implicated in the development of endothelial damage, hypertention, coronary spasm and myocardial infarction [2,3]. The gene encoding eNOS is located on human chromosome 7q36, a genetic region previously linked to the metabolic syndrome, cardiovascular and renal disease risk factors [4-5].

Several polymorphisms have been identified in the eNOS gene. A single nucleotide polymorphism (SNP), -786T>C, has been found in the 5'-flanking region of the eNOS gene involving a substitution of thymine (T) to cytosine (C) at a locus up stream of eNOS [6,7]. Another common variant of the eNOS has a G to T transversion at nucleotide position 894 (894 G>T), which leads to a change of amino acid at position 298 (Glu298Asp) [8,9]. A 27-bp repeat polymorphism in intron 4 of this gene has been associated with variation in plasma levels of nitrite and nitrate (NOx)-stable metabolites of NO [10,11]. This polymorphism was associated with myocardial infarction (MI) and predicted smoking-dependent risk of CAD [12,13]. It has been shown to segregate with lower plasma metabolites in non-pregnant Japanese females [11]. Besides, this polymorphism was linked to recurrent miscarriage in Caucasians [14]. However, other studies have demonstrated no association between this repeated polymorphism and CAD [15,16].

In fact information available on association of eNOS intron 4b/a VNTR polymorphism with CAD is controversial.

In the present study, we have investigated the association between the 4a4b polymorphism of the eNOS gene and the presence of CAD.

We have also evaluated the relationship between NO concentrations, the 4a4b polymorphism and the risk of CAD in the Tunisian population.

Materials and Method

Study population

A total of 436 unrelated Tunisian subjects were into this case-control study, of which 274 were CAD 162 healthy controls. CAD patients enrolled from the Department of Cardiology, Farhat Hached University Hospital of Sousse. Controls were enrolled providing that they had neither a history nor clinical or instrumental evidence of atherosclerosis in peripheral vascular districts.

All participants were interviewed; data on smoking habits, hypertension, diabetes, dyslipidemia and family history of CAD were recorded. Informed consent was obtained from all patients and controls according to the guidelines of our ethics committee.

Weight and height were measured on the subjects barefooted and lightly clothed. Body mass index (BMI; kg/m^2) was calculated and obesity was defined as BMI>30 kg/m^2 [17]. Diabetes mellitus was defined hyperglycemia, requiring antidiabetic drugs or testing blood glucose over 7.0 mmol/L. Hypertension was defined as systolic blood pressure ≥ 140 mmHg and/or diastolic blood pressure ≥ 90 mmHg, or the use of antihypertensive drug treatement, or a combination of these. Dyslipidemia was defined as total cholesterol (TC) level>6.47 mmol/L and /or triglyceride (TG) level>2.26 mmol/L. A smoker was defined as a current smoker or an ex-smoker.

Blood samples were obtained from all subjects after 12h fasting and placed in EDTA tubes and stored at -20°C until the time of assay.

Biochemical analysis

The serum concentrations of triglyceride (TG), total cholesterol, LDL cholesterol (LDL-C) and HDL cholesterol (HDL-C) were measured by the standard methods used in the clinical laboratory of the hospital.

DNA analysis

Genomic DNA was extracted from peripheral blood leukocytes by standard method (promega kit, USA). The extracted genomic DNA was stored at 4°C until analysis. The genotyping of the eNOS 4b/a polymorphism was accomplished by the method of Wang et al (10) with minor modifications. Brievely, a genomic DNA fragment was amplified by the polymerase chain reaction (PCR) using oligonucleotide primer pairs that flank the 27-bp direct repeat region in intron 4 of that eNOS. Primer pairs for PCR were as follows: sense 5'-AGG CCC TAT GGT AGT GCC TTT-3' and antisense 5'-TCT CTT AGT GCT GTG GTC AC-3'.

The PCR was performed in a 25-µL final volume that contained 100-ng genomic DNA, 20 mM each primer, 10 mM of each deoxyribonucleotide triphosphate (dNTP), 25mM Mgcl2, 5 µl of 10x PCR buffer and 1U of DNA Taq Polymerase (promega corporation, USA). The PCR mixtures were heated to 94°C for 5 minutes for denaturation and underwent 30 cycles at 94°C for 1 minute for denaturation, 57°C for 1 minute for annealing and 72°C for 1minute for extension. Finally, extension was conducted at 72°C for 10 minutes.

The amplified fragments were separed on 2% agarose gels with ethidium bromide staining. A 420-bp band indicated five repeats of the 27-bp (eNOS4b allele), and a 393-bp band four repeats (eNOS4a allele).

The eNOS4b allele is the common allele.

Determination of plasma NOx concentrations

Since NO is unstable and quickly auto-oxidized to nitrite and nitrate, the plasma NOx concentrations were measured a total nitrite concentration.

Brievly, a 100 µl of plasma sample was reacted to deproteinization with 30 µl of zinc sulfate (0.5 g/ml), and the resulting sample was centrifuged at 4000 x g at 4°C for 5 min. A 75 µl of supernatant was applied to a microtiter plate well and followed by the addition of 75µl of Griess reagent (1 g/l sulfanilamide, 0.1 g/l N-[1-naphthyl]ethylenediamine and 5 g/l phosphoric acid). After color development at 25°C for 10 min, the absorbance was measured on a microplate reader at 540 nm. Each sample was assayed in duplicate wells. The absorbance at 620 nm was used as background values and the calibration curve was plotted using known amounts of potassium nitrate in distilled water. The linearity was observed from 0 to 100 µmol/l.

Statistical analysis

All statistical analyses were performed with SPSS V.10. The differences between groups were examined by ^{2} test or an independent student t-test whichever was appropriate. Allele frequencies were estimated by the gene counting method. The frequencies of the alleles and genotypes were compared between patient and control group by the ^{2} test when appropriate. The odds ratio (OR) and 95% confidence interval (CI) were also estimated. The ^{2} test was used for deviation of genotype distribution from Hardy-Weinberg equilibrium. Logistic regression analysis was used to assess the independent effect of each risk factor on the presence of CAD.

The plasma NOx concentrations were normalized for analyses by non-parametric test (test of Kruskal-Wallis and median) because of their skewed statistical distribution. The association between plasma NOx concentrations and other contributing factors was evaluated by the multiple regression analysis with forward stepwise selection. P< 0.05 was considered to be significant.

Results

Table 1 summarizes the clinical and biochemical data of CAD patients and the control subjects. There are no differences in the HDL, TG, homocysteine, CPK and NOx between the two groups. While the prevalence of mean age, gender, BMI, hypertension, diabetes mellitus, LDL and smoking were significantly higher in patients group than the control group (p<0.001). The CAD group showed higher concentrations of Total-C and creatinine compared with the control group.

Characteristic	Patients	Controls	P
Male/Female, n%	158/ 108	68/94	0

Age	64,65 ± 10.69	53,76 ± 7.44	0
BMI	28.79 ± 4.47	26.61 ± 3.58	
Hypertention, n%	161 (58.7%)	10 (6.17%)	0
diabetes, n%	164 (59.8%)	32 (17.7%)	0
smoking, n%	94 (34.3%)	29 (17.9%)	0
Total-C (mmol/l)	4.65 ± 1.19	4.91 ± 1.01	0.039
LDL-C (mmol/l)	2.18 ± 1.08	2.78 ± 0.99	0
HDL-C (mmol/l)	1.18 ± 0. 46	1.28 ± 0.35	0.102
TG (mmol/l)	2.45 ± 7.278	1.63 ± 1.20	0.212
Homocysteine (µmom/l)	21.39 ± 25.89	28.37 ± 56.26	0.334
Creatinine (µmom/l)	100.13 ± 58.04	82.08 ± 63.83	0.009
CPK (U/l)	271.31 ± 432.93	82.21 ± 65.17	0.059
NO (µmol/l)	8.75	5.49	0.123
median			
Data are expressed as means ± SD and as percent in other variable.			

Table 1: Demographic and clinical characteristics of coronary artery disease patients and controls.

Two alleles of the VNTR in the human eNOS gene were detected in Tunisian subjects (Figure 1). One allele contained four of 27bp repeats (eNOS 4a allele) giving rise to a PCR product of 393 bp, whereas the other contained five of such repeats (eNOS 4b allele), yielding a PCR product of 420 bp.

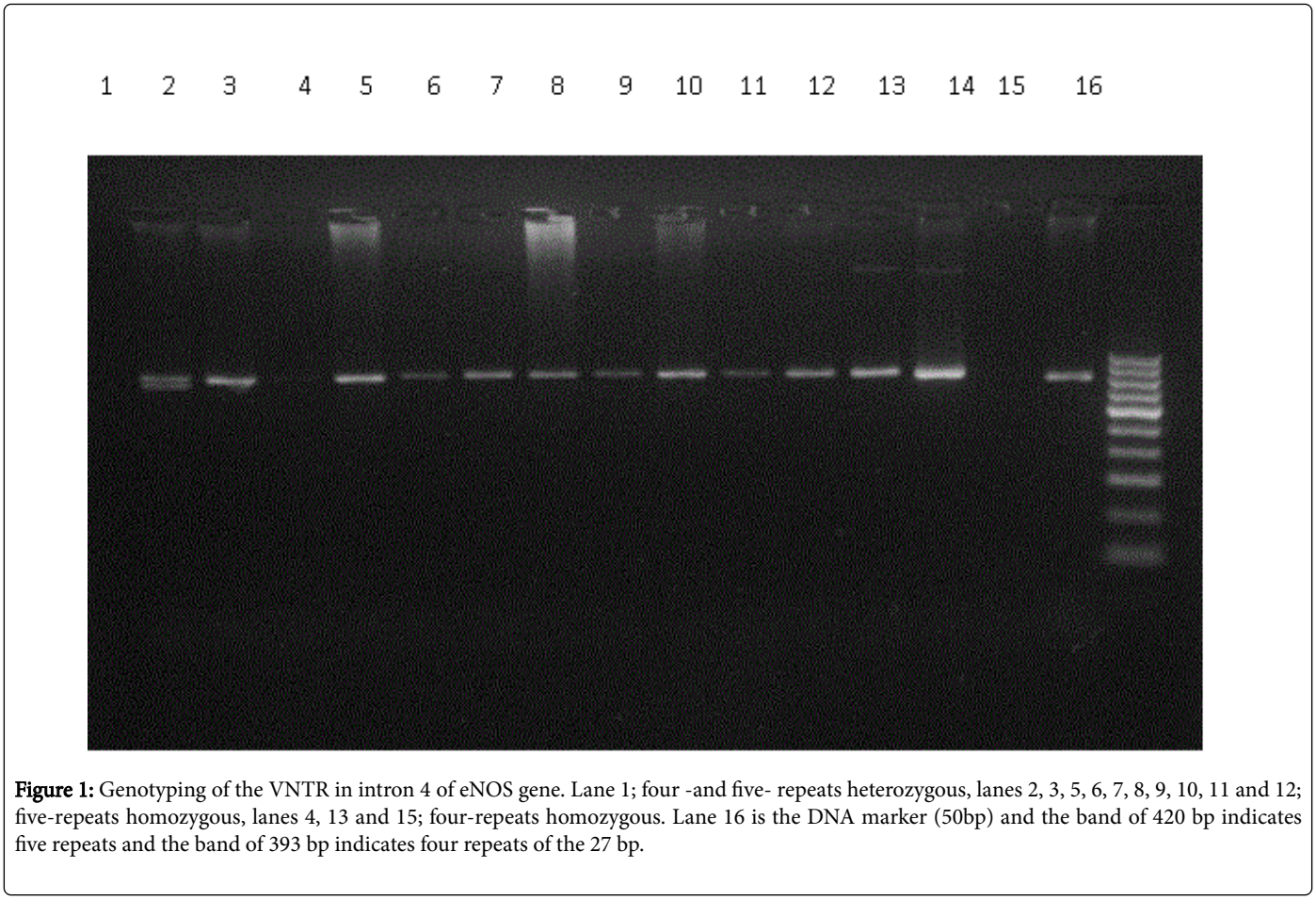

Figure 1: Genotyping of the VNTR in intron 4 of eNOS gene. Lane 1; four -and five- repeats heterozygous, lanes 2, 3, 5, 6, 7, 8, 9, 10, 11 and 12; five-repeats homozygous, lanes 4, 13 and 15; four-repeats homozygous. Lane 16 is the DNA marker (50bp) and the band of 420 bp indicates five repeats and the band of 393 bp indicates four repeats of the 27 bp.

The polymorphism of the eNOS gene 4a4b was analysed and the genotypes distributions and alleles frequencies in CAD patients and controls are shown in Table 2.

	CAD (n=274)	Normotensive (n=162)	Unadjusted OR a	p a	Multi-adjusted ORb	Pb
Genotypes:						
4b4b	84 (33.7%)	100 (63.3%)	1	-	1	-
4a4b	159 (63.9%)	48 (30.4%)	1.4 [0.48-4.01]	0.53	1.76 [0.48-6.48]	0.39
4a4a	6 (2.4%)	10 (6.3%)	5.52 [1.90-15.97]	0.002	4.54 [1.22-16.8]	0.023
4b4b	84 (33.2%)	101 (63.9%)	3.56 [2.34-5.40]	0	0.402 [0.23-0.67]	0.001
4a4b+4a4a	169 (66.8%)	57 (36.1%)				
Alleles						
4b	327 (65.66%)	248 (78.48%)	1.9 [1.37-2.64]	0		
4a	171 (34.34%	68 (21.52%)				

CAD, Coronary Artery disease; OR, odds ratio; CI, Confidence interval. a Crude logistic regression model, b Adjusted for diabetes, hypertension, dyslipidemia and smoking.

Table 2: Distribution of genotype and allele frequencies adjusted and unadjusted odds ratio and 95% confidential intervals of the presence of CAD among eNOS 4a4bgenotypes.

The distribution of the eNOS genotypes and alleles and control groups were compatible with Hardy-Weinberg equilibrium. The frequencies of bb, ab, aa genotypes of 4 VNTR polymorphism were 33.7%, 63.9%, 2.4%, respectively, for the patient group and 63.3%, 30.4%, 6.3%, respectively, for the control group. Alleles frequencies for the 4 VNTR polymorphims were among patients and controls 65.66% and 78.48%, respectively, for the most frequent allele b (wild type) and 34.34% and 21.52%, respectively, for allele a. The statistical analysis showed that there is a significant difference in the genotype frequencies (OR=3.56 [2.34-5.4], p=0.001) and allele frequencies (OR=1.9 [1.37-2.64], p<0.001) between CAD patients and controls subjects.

Correction for smoking, diabetes, dyslipidemia and hypertension strengthened the estimate (Table 2).

We used binary logistic regression to test for independent correlates of the presence of CAD. Included in the model were smoking, diabetes, dyslipidemia and hypertension and the eNOS 4a4b polymorphism. Hypertension (p<0.001), diabetes mellitus (p<0.001), smoking (p<0.003) and the eNOS4a4b polymorphism were independent correlates of the presence of hypertension (Table 3).

Variables	Beta-coefficient	SE	P value	OR (95% CI)
Hypertention,	-2.715	0.381	<0.001	0.066[0.03-0.14]
Diabetes	-1.489	0.291	<0.001	0.22[0.128-0.39]
Smoking	-6.41	0.213	0.003	0.52 [0.34-0.8]
Dyslipidemia	0.125	0.299	0.67	1.13 [0.61-2.03]
eNOS 4a4b				
4a4b	0.569	0.663	0.39	1.76 [0.48-6.48]
4a4a	1.514	0.667	0.023	4.54 [1.22-16.81]
Constant	7.467	1.141		<0.001
OR=odds ratio, CI=confidence interval, SE=standard error.				

Table 3: Binary logistic regression modeling using CAD as dependant variable (n=436).

In males and females separately (Table 4), the odds ratio of CAD for 4a4b genotype was 2.54 [1.4-4.62] at 95% CI, p=0.002 in males and 5.29 [2.88-9.71], p<0.001 in females.

	CAD (n=274)	Normotensive (n=162)	Unadjusted OR a	pa	Multi-adjusted OR b	Pb
Female						
Genotypes:						
4b4b	29 (27.4%)	61 (63.3%)	1	-	1	
4a4b	74 (69.8%)	24 (26.1%)	1.1 [0.26-4.60]	0.88	0.68 [0.11-3.93]	
4a4a	3 (2.8%)	7 (7.6%)	7.19 [1.72-30.02]	0.007	3.00 [0.51-17.49]	
4b4b	29 (27.1%)	61 (63.3%)	5.29 [2.88-9.71]	0	0.25 [0.10-0.61]	
4a4b+4a4a	78 (72.1%)	31 (33.7%)				
Alleles						
4b	132 (62.26%)	146 (79.34%)	2.32 [1.48-3.66]	0		
4a	80 (37.74%)	38 (20.66%)				
Male						
Genotypes						
4b4b	55 (38.5%)	39 (59.1%)	1	-	1	
4a4b	85 (59.4%)	24 (36.4%)	1.41 [0.27-7.35]	0.68	3.7 [0.47-28.99]	
4a4a	3 (2.1%)	3 (4.5%)	3.54 [0.67-18.68]	0.13	6.62 [0.84-52.2]	
4b4b	55 (37.7%)	40 (60.6%)	2.54 [1.4-4.62]	0.002	0.53 [0.26-1.06]	
4a4b+4a4a	91 (62.3%)	26 (39.4%)				
Alleles						
4b	195 (68.18%)	102 (77.27%)	1.58 [0.98-2.55]	0.056		
4a	91 (31.82%	30 (22.73%)				
CAD, Coronary Artery disease; OR, odds ratio; CI, Confidence interval.						

a Crude logistic regression model

b Adjusted for diabetes, hypertension, dyslipidemia and smoking.

Table 4: Gender wise distribution of genotype and allele frequencies adjusted and unadjusted odd ratios and 95% confidential intervals of the presence of CAD among eNOS 4a4b genotypes.

Serum NO values were not correlated with genotypes of 4a4b of the eNOS gene in CAD patients and controls. In the control group, mean serum NO concentrations were 5.49μM. In CAD patients, means levels were 8.75 μM (Table 5). There was no significant difference among the three 4a4b genotypes in both controls and patients (p>0.05).

Group	Total No	bb	ab	aa	median e	P
		n NO (μM)	n NO (μM)	n NO (μM)		
Controls		38	18	1	5.49	0.31
CAD patients		42	93	4	8.75	0.57

Table 5: Comparison of eNOS 4a4b genotypes with Mean serum NO Levels among CAD group and control subjects.

Discussion

This study investigated the association between eNOS gene intron 4a4b VNTR polymorphism and CAD in Tunisian patients and controls. Our results showed that a allele frequency of the eNOS gene intron 4a4b VNTR polymorphism was significantly higher in patients with CAD. This association persisted after adjusting the confounding factors for several potentials. The 4a allele frequency of the eNOS gene 4a4b polymorphism was considerably higher in patients with CAD than in controls. Many researchers have dealt with the correlation between the eNOS gene 4a4b polymorphism and CAD. They obtained conflicting results; some of them have shown such association, while others have not.

The relationship between 4a4b genotype and CAD was first desribed by Wang et al. in Australia [13]. It was observed in Japanese and European patients with acute myocardial infarction (MI) [18,19]. The 4a4b genotype frequencies noticed in our groups are similar to that obtained by Wang et al [13].

However, other investigators did not detect a link with CAD [20-23]. In fact, the mechanism by which the eNOS 4a4b polymorphism confers susceptibility to MI has not been yet understood. The 4a4b polymorphism is associated with altered plasma NO levels, influencing both NO and the enzyme production [11]. Several studies have reported that lower NO plasma levels for 4a allele curriers are observed in Asians and African-Americans but not in others [24]. In the present study, a lack association between eNOS genotypes and serum NO level in CAD patients and controls is evident. Our results are consistent with the studies done on Turkish and Egyptian population which showed no significant difference in serum NO values among the three Glu298Asp genotypes in both controls and patients [25,26]. Yoon et al [27] proved that only in the control group, plasma NO was considerably dependent on the genotypes of Glu298Asp polymorphism; this relationship was not observed in CAD patients. Two interpretations can be proposed for such ambiguous results. First, the discrepancy may stem from the nature of the sampling variability in case-control design. Second, it reflects different genetic backgrounds between the two ethnic groups. The current study has assessed these interpretations but it has not provided evidence for the significant association with the eNOS4a4b polymorphism.

Furthermore, data from the literature have reported that the variance of NO circulating levels accounted for 25% [10]. It has been demonstrated in a Korean population that the effect of the 4a4b polymorphism on the variance of plasma NO depends on the smoking status [28]. It was previously hypothesized that the latter might explain two incompatible reports of either higher or lower plasma NOx concentrations in subjects with eNOS 4a variant [29]. Our data does not support the previous one [29] with respect to the smoking status.

Besides, it is equally important to understand that smoking and/or its constituent(s) are likely to be associated with in vivo inflammatory action resulting in the inducible NOS (iNOS) expression [30]. Actually, smoking subsequently marked an increase in plasma NOx concentration. However, this sequential pathway activating iNOS occurs transiently as far as it is in a morbid situation. Otherwise, eNOS activation influences plasma NOx concentrations in basal state [11].

At the present time, we do not know if smoking confers such a morbid situation; even so, we do not still know whether the increased plasma NOx concentrations are maintained continuously without degradation or not. Previous studies have shown that measurement of NOxin blood collected after at least 12 h can reliably reflect basal (endogenous) NO production [11,31,32]. In fact, our study shows that the no differences in NO production between the controls and patients is due to the time of blood collection done after at least 12 h.

Thus, further detailed experiments need to be conducted for reaching clear conclusion.

The discrepancy between studies could be caused not only by the differences in ethnicity or different a sample sizes, but most importantly by the different selection criteria adopted for patients and controls, especially clinical presentation, age, race, geographic area and environmental risk factors [33].

Some limitations of our work should be taken into consideration. First, this work is based on a limited number of patients and controls. Second, the coronary angiography was not performed in control subjects who had no symptoms and no vascular events history of any form.

Finally, we did not measure eNOS activity and a large sample should be examined to confirm the relation between polymorphism and plasma NOx concentrations.

In conclusion, we demonstrated that the 4a4b polymorphism of the eNOS gene is significantly and independently associated with CAD, but no significant difference was observed between eNOS genotypes and NOx concentrations in CAD patients and controls in the Tunisian population.

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