

eNOS gene variants and the risk of premature myocardial infarction

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Abstract.

BACKGROUND: Endothelial nitric oxide synthase (eNOS) as well as nitric oxide play an important role in the regulation of cardiovascular function. There are limited and controversial data regarding the impact of polymorphisms of eNOS gene that is implicated in the vasoconstrictive properties of the endothelium in the pathogenesis of premature myocardial infarction (MI).

OBJECTIVE: We examined whether two common polymorphisms of eNOS gene (G894T and T786C) are associated with the development of premature MI.

METHODS: We recruited 107 patients with premature MI and compared them to 103 age- and sex- matched controls. All patients underwent coronary angiogram and were classified into the subgroup of patients with 'normal' or 'near normal' coronary arteries and the subgroup of patients with significant coronary artery disease ($\geq 50\%$ stenosis in lumen diameter of coronary arteries). The genetic polymorphisms of eNOS gene were assayed with polymerase chain reaction and reverse hybridization.

RESULTS: Nineteen patients (17.8%) had 'normal' or 'near normal' coronary arteries. A significantly higher frequency of homozygosity for the 786C (32%) and the 894T (21%) alleles of the eNOS gene in patients who develop early MI in the setting of angiographically 'normal' or 'near normal' coronary arteries were found.

CONCLUSIONS: Our data suggest that the T786C and the G894T genetic polymorphisms are associated with the development of MI in very young individuals, whose coronary arteries are characterized by very small atheromatic burden.

Keywords: Cardiovascular disease, genetic polymorphisms, early myocardial infarction, endothelial nitric oxide synthase

1. Introduction

It is well established that endothelial function is critical for vascular homeostasis. This is mediated via production of a number of biochemical mediators with vasodilatory or vasoconstrictive properties [4,11,18]. Endothelial – derived nitric oxide (eNO) produced by endothelial nitric oxide synthase (eNOS) plays a pivotal role in the preservation of the endothelium homeostasis, regulation of vascular tone and endothelial-leukocyte interaction [3,26].

The gene encoding eNOS is located on chromosome 7 (7q35-36) [7,13]. Several polymorphisms of the eNOS gene have been reported so far but their impact on eNO production and expression is not yet clarified. The genetic polymorphisms *G894T* and *T786C* are the most clinically relevant polymorphisms in the eNOS gene that have been described [2,13]. However, the *G894T*(Glu298Asp) and *T786C* (a mutation located in the 5-flanking region of the eNOS gene) genetic polymorphisms seem to be implicated in the development of coronary heart disease (CHD) [8,9,14,17,25]. Previous studies have shown that these polymorphisms are associated with reduced eNO generation [7,9,13]. Furthermore, they may be implicated in the development of premature myocardial infarction

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(MI), although their actual role has been questioned by several studies [1,2,6,15,19,25].

Young adults constitute a relatively small percentage of patients who sustain MI. It is estimated that young patients make up between 5% and 10% of all MIs [10,16,21,23]. Young patients with MI share a different risk factor profile compared to older patients. In particular, MI at young ages is usually associated with less atheromatic burden in coronary arteries and higher prevalence of smoking and family history of CHD [22, 24]. Another distinct characteristic of patients with MI at young age is the relatively high proportion of angiographically 'normal' or 'near normal' coronary arteries [28]. This suggests that the impact of other pathogenetic mechanisms such as coronary spasm may have more pronounced role in the development of premature MI [10,22].

In the present study, we studied the prevalence of the *G894T* and *T786C* genetic polymorphisms in young survivors (≤ 35 years) of MI in order to estimate their impact on the development of premature MI.

2. Materials and methods

The study population consisted of 107 patients who had survived their first MI occurring ≤ 35 years of age. They had been admitted to the Coronary Care Unit of the General Hospital of Nikea and the University General Hospital Attikon between January 2003 and December 2010. The diagnosis of MI was based on the presence of ≥ 2 of the following three criteria: (1) characteristic chest pain lasting > 30 min, (2) ST elevation > 0.1 mV on at least 2 adjacent electrocardiographic leads and (3) increase of creatine kinase (CK) to peak levels of at least 2-fold the upper limit of normal values [20].

We also recruited 103 healthy age- and sex-matched subjects who had undergone a minor orthopedic intervention in the University General Hospital Attikon who served as the control group. All control subjects had no personal or family history of cardiovascular disease. The study was approved by the ethics committee of our institution and all subjects gave their informed consent.

All participants were interviewed and special attention was paid on reporting cardiovascular risk factors and use of medication. The following definitions were used: hypertension, blood pressure $\geq 140/90$ mmHg and/or antihypertensive treatment; hypercholesterolemia, total cholesterol > 200 mg/dl

and/or lipid lowering medication. Smokers from the patients group were considered those who smoked until MI and from the controls those who reported smoking currently and regularly (at least five cigarettes per day).

2.1. Coronary angiography

All patients underwent coronary angiography and left ventriculography by the Judkins technique. According to angiographic findings they were divided into two subgroups. The first subgroup consisted of patients in whom epicardial coronary arteries had smooth contours and no focal diameter reduction ('normal') or coronary arteries with non-haemodynamically significant atherosclerotic lesions ($< 50\%$ stenosis) ('near normal'). The second subgroup was angiographically defined as $\geq 50\%$ stenosis in lumen diameter of coronary arteries (significant CHD).

2.2. Blood sampling and biochemical analysis

Peripheral blood samples were collected from patients and controls after overnight fasting for assessing levels of lipids and genotyping. In particular, blood from patients was collected within 24 h from admission.

Total cholesterol, triglycerides as well as High Density Lipoprotein (HDL) cholesterol levels were determined using enzymatic colorimetric assays (Schiaffarelli Biosystems Inc., USA). Low Density Lipoprotein (LDL) cholesterol was calculated by the Friedewald equation [12].

2.3. DNA analysis

Genomic DNA was extracted from EDTA anticoagulated blood by using standard methods (CVD strip assay A, Viennalab, Austria). The presence of genetic polymorphisms of the eNOS gene (*G894T* and *T786C* polymorphisms), was assayed by polymerase chain reaction (PCR) and amplification of the target sequence using biotinylated primers (*T786C* polymorphism: 5' CACCTGCATTCTGGGAAGCTGTA 3' & 5' GCCGCAGTAGCAGAGAGAC 3'; *G894T* polymorphism: 5' GGCACCTCAAGGACCAGCT 3' & 5' ACG-GCTGGACCCAGG 3'). The PCR cycles were optimized as follows: 2 min of initial denaturation at 94°C, followed by 35 cycles of amplification (15 sec denaturation at 94°C, 30 sec annealing at 58°C, 30 sec extension at 72°C) and final extension of 3 min at 72°C.

Table 1
Prevalence of traditional risk factors in young survivors of myocardial infarction

	Total	Patients (n = 107)		Controls (n = 103)	p			
		Significant CHD (n = 88)	'Normal' or 'near normal' (n = 19)		P [#]	P [*]	P [†]	P [‡]
Age (year)	32.1 ± 3.1	32.5 ± 3.4	31.4 ± 2.3	31.8 ± 3.2				
Current smokers (%)	95.2	96.3	94.1	47.6	< 0.001	< 0.001	< 0.001	NS
Hypertension (%)	9.8	12.6	5.0	1.9	< 0.001	< 0.001	0.025	< 0.001
Total cholesterol (mg/dl)	238.2 ± 71	251.8 ± 68	224.8 ± 50	189.6 ± 24	< 0.001	< 0.001	0.01	NS
Triglycerides (mg/dl)	170.9 ± 90	165.2 ± 86	175.6 ± 125	107 ± 63	< 0.001	< 0.001	< 0.001	NS
LDL cholesterol (mg/dl)	156.1 ± 58	174.6 ± 70	130.6 ± 45	119.4 ± 27	< 0.001	< 0.001	NS	< 0.001
HDL cholesterol (mg/dl)	33.5 ± 11.7	32.6 ± 11.6	34.3 ± 14	50 ± 8.5	0.02	0.015	0.025	NS

[#] P patients vs. controls; ^{*} P patients with significant CHD vs. controls; [†] P patients with 'normal' or 'near normal' coronary arteries vs. controls; [‡] P patients with significant CHD vs. patients with 'normal' or near 'normal' coronary arteries; NS Not Significant; ± SD.

The amplification products were hybridized to a test strip containing allele-specific oligonucleotide probes (T786C polymorphism wild type: 5' CAAGCTCTTC-CCTGGCAG 3' & mutant: 5' CAAGCTCTTCCCTG-GCGG 3'; G894T polymorphism wild type: 5' TGGG-GGCTCATCTGGGGC 3' & mutant: 5' TGGGGGAT-CATCTGGGGC 3') immobilized as an array of parallel lines. Bound biotinylated sequences were detected using streptavidin-alkaline phosphatase and specific color substrate (CVD strip assay A, Viennalab, Austria).

2.4. Statistical analysis

Quantitative values are expressed as median (S.D.) and were compared using unpaired Student's t-test. For repeated measurements comparisons between groups were initially tested with Freedman analysis of variance. Discontinuous variables were tested by a contingency χ^2 test. A p value < 0.05 was considered significant. The SPSS version 13 (SPSS Inc., Chicago, USA) statistical package was used.

3. Results

3.1. Traditional risk factors

CK peak levels during the acute phase of MI were 2455 ± 2240 U/ml. The prevalence of traditional risk factors for MI and biochemical measurements are shown in Table 1. There was a significantly higher prevalence of smoking and hypertension in patients than in controls. Total cholesterol, triglycerides and LDL cholesterol levels were also significantly higher, while HDL cholesterol levels were significantly lower in patients compared to controls.

Patients with MI were further divided into two sub-groups according to coronary anatomy ('normal' or 'near normal' coronary arteries and significant CHD) as shown in Table 1. The frequency of smoking and hypertension as well as the levels of total cholesterol and triglycerides remained significantly higher in both sub-groups compared to controls. LDL cholesterol levels remained significantly higher only in the patients with MI significant CHD compared to controls, while HDL cholesterol levels were significantly lower in both sub-groups compared to controls.

3.2. eNOS polymorphisms

3.2.1. T786C polymorphism

The prevalence of homozygosity for the C allele was significantly higher in patients compared to controls (16% vs. 6%, $p < 0.001$) (Table 2). There was a higher, although not statistically significant, prevalence of CC homozygosity in patients with MI and significant CHD than in control subjects (12% vs. 6%, $p = 0.06$), as well as the prevalence of CC homozygosity was significantly higher in patients with 'normal' or 'near normal' coronary arteries compared to controls (32% vs. 6%, $p < 0.001$) (Table 2).

3.2.2. G894T polymorphism

The prevalence of homozygosity for the T allele was significantly higher only in patients with 'normal' or 'near normal' coronary arteries compared to controls (21% vs. 10%, $p < 0.001$) (Table 3).

4. Discussion

It is well established that the majority of patients who develop MI show significantly higher values of at least one of the traditional risk factors (smoking, hy-

Table 2
Prevalence of T786C polymorphism in patients and control subjects

T786C polymorphism	Total	Patients (n = 107)			p			
		Significant CHD (n = 88)	'Normal' or 'near normal' (n = 19)	Controls (n = 103)	P [#]	P [*]	P [†]	P [‡]
TT	41(38%)	35(40%)	6(32%)	70(68%)	< 0.001	< 0.001	< 0.001	NS
TC	49(46%)	42(48%)	7(36%)	27(26%)	< 0.001	< 0.001	NS	NS
CC	17(16%)	11(12%)	6(32%)	6(6%)	< 0.001	NS	< 0.001	< 0.001
Allele frequency								
T	131(61%)	112(64%)	19(50%)	167(81%)	0.03	NS	< 0.001	0.04
C	83(39%)	64(36%)	19(50%)	39(19%)	< 0.001	< 0.001	< 0.001	0.04

P patients vs. controls; *P patients with significant CHD vs. controls; †P patients with 'normal' or near 'normal' coronary arteries vs. controls; ‡P patients with significant CHD vs. patients with 'normal' or 'near normal' coronary arteries; NS Not Significant.

Table 3
Prevalence of G894T polymorphism in patients and control subjects

G894T polymorphism	Total	Patients (n = 107)			p			
		Significant CHD (n = 88)	'Normal' or 'near normal' (n = 19)	Controls (n = 103)	P [#]	P [*]	P [†]	P [‡]
GG	50(47%)	43(49%)	7(37%)	50(49%)	NS	NS	NS	NS
GT	46(43%)	38(43%)	8(42%)	42(41%)	NS	NS	NS	NS
TT	11(10%)	7(8%)	4(21%)	11(10%)	NS	NS	< 0.001	0.02
Allele frequency								
G	146(68%)	124(70%)	22(58%)	142(69%)	NS	NS	NS	NS
T	68(32%)	52(30%)	16(42%)	64(31%)	NS	NS	0.04	NS

P patients vs. controls; *P patients with significant CHD vs. controls; †P patients with 'normal' or 'near normal' coronary arteries vs. controls; ‡P patients with significant CHD vs. patients with 'normal' or 'near normal' coronary arteries; NS Not Significant.

pertension, hyperlipidaemia) associated to atherosclerosis. However, almost 20% of very young patients with MI appear with 'normal' or 'near normal' arteries. In our study, we investigated the hypothesis that genetic factors that affect pathways such as endothelial function and coronary artery spasm might trigger the development of MI in young patients who have no or minor atheromatic burden as proved by angiographic data. We evaluated the frequency of two common polymorphisms of the eNOS gene (*G894T*, *T786C*) associated with reduced production of eNO and ineffective vasodilation in a group of very young patients with MI and we further divided this group in two subgroups according to angiographic data of the patients. We found that patients who develop premature MI in the setting of angiographically 'normal' or 'near normal' coronary arteries present with higher frequency of homozygosity for the *T786C* and the *G894T* genetic polymorphisms of the eNOS gene.

Previous studies have reported conflicting results regarding the association of the genetic polymorphisms of the eNOS gene with the risk of MI. Nakayama et al. reported that the frequency of the *T786C* polymorphism was significantly higher in MI patients with no stenosed vessels compared to those with stenosed vessels and concluded that this polymorphism is associated with the development of MI in patients without significant CHD [19]. Granath et al. found no signifi-

cant difference between patients and controls regarding frequency of *CC* (mutant) genotype [15]. Additionally, Ciftçi et al. showed that the high frequency of *TT* (wild type) genotype in the patients may support no relationship of *T786C* polymorphism with premature MI [6].

Similar conflicting results concerning the development of premature MI were obtained for the *G894T* genetic polymorphism. Spence et al. observed that individuals, who were homozygous for the mutant allele (*TT* genotype), were not associated with an increased risk of MI [25]. Antoniadis et al. in an elegantly designed study with 229 patients with premature MI demonstrated that homozygosity for *G894T* polymorphism is associated with a significantly increased risk for premature MI [2]. Andrikopoulos et al. reported that the frequency of the *G894T* polymorphism was found to be significantly higher in MI patients [1]. Similarly, Isordia-Salas et al. showed that the Glu298Asp polymorphism represents an independent risk factor for premature MI [17].

The unique feature of our study was the recruitment of patients who suffered MI very early in their life (≤ 35 years). By lowering the age limit we formed a subgroup of young patients with specific characteristics. The atheromatic burden in coronary arteries in this age group is relatively low as this is reflected by the relatively high prevalence of 'normal' or 'near normal' coronary angiograms. In our young population 17.8%

of the patients had angiographically 'normal' or 'near normal' coronary arteries. By comparison, this prevalence is < 5% in older population [28]. Moreover, in young age the presence of other traditional cardiovascular risk factors is uncommon with the exception of smoking and hyperlipidemia.

The precise mechanism of development of premature MI is unknown. The low atheromatic burden of coronary arteries in the first four decades of life allows us to hypothesize that mechanisms such as hypercoagulable state, coronary artery spasm and inflammation per se or in combination may play a role in the pathogenesis of premature MI. Uncompromised production of eNO leading to ineffective vasodilation and thus increased possibility of spasm might constitute a candidate pathogenetic mechanism for the development of MI in this group of patients. Therefore, the study of polymorphisms in the eNOS gene associated with reduced eNO production might be of interest in this group of patients. Implication of polymorphisms involved either in the regulation of expression (*T786C*) or in post-translational modification of eNOS (*G894T*) was therefore investigated in a group of very young patients with MI.

In particular, homozygosity for *T786C* polymorphism has been reported to produce a significant reduction in the eNOS gene promoter activity and thus eNO production [13]. Moreover, it has been suggested that homozygosity for *G894T* polymorphism leads to susceptibility to proteolytic cleavage in endothelial cells and vascular tissues and results in reduced levels of functional eNOS and subsequently reduced levels of eNO [9].

Few limitations of our study have to be addressed. The results may be biased since we recruited only survivors of MI. As a result, patients who died before arriving at hospital might have had a different pattern of the above studied polymorphisms. Another limitation is the relatively small size, for a genetic study, of our population.

In conclusion, our study shows that the *T786C* and the *G894T* genetic polymorphisms seem to be associated with the development of MI in very young individuals whose coronary arteries are characterized by very small atheromatic burden. However, large studies need to be undertaken in young populations in order to further elucidate the gene-environment interrelation that underlies the pathophysiology of premature MI.

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