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Gene-Gene Interaction of ACE I/D, Endothelial Nitric Oxide Synthase 4 a/b and ApoE does not Affect Coronary Artery Disease Severity

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A – research concept and design; **B** – collection and/or assembly of data; **C** – data analysis and interpretation; **D** – writing the article; **E** – critical revision of the article; **F** – final approval of article; **G** – other

Abstract

Objectives. Previous studies have shown the impact of angiotensin converting enzyme (ACE) insertion/deletion (I/D), endothelial nitric oxide synthase (eNOS) polymorphisms and ApoE genotypes on coronary artery disease (CAD). The aim of this study is to investigate the relationship between the genetic polymorphisms and the severity of CAD and to evaluate their potential interactions.
Material and Methods. All patients underwent coronary angiography; coronary score (CS) and severity score (SS) were calculated for them. ACE I/D, eNOS and ApoE polymorphisms were detected by polymerase chain reaction (PCR).
Results. Neither CS nor SS showed a direct relationship with eNOS and ApoE genotypes. CS and SS were found to be high in patients carrying the ACE DD allele ($p = 0.034$ and $p = 0.009$). In the gene interactions, there was an increase in the SS only in patients with coexisting eNOS b/b genotype and ACE DD allele ($p = 0.043$).
Conclusions. The interactions of the gene polymorphisms investigated don't play an important role in determining an individual's risk for the severity of CAD (**Adv Clin Exp Med 2014, 23, 2, 215–223**).

Key words: ACE gene, ApoE gene, eNOS gene, gene–gene interaction, severity of coronary artery disease.

Coronary artery disease (CAD) is the most common cause of death in the world. It is a complex disease with both environmental and genetic determinants. Genetics, in addition to other well-known major risk factors, is an important mechanism in the development of CAD [1–7]. Numerous studies have indicated an association of CAD with gene polymorphisms [1–5].

Angiotensin converting enzyme (ACE) is a member of the renin-angiotensin-aldosterone system, which converts angiotensin I to angiotensin II. The studies on the association of ACE gene insertion/deletion (I/D) polymorphism with CAD have shown that the DD genotype is associated with increased risk of developing CAD [3–5]. In addition, nitric oxide (NO) derived from the endothelium is considered as an important mediator, and synthesized from L-arginine by the enzyme endothelial nitric oxide synthase (eNOS). Numerous studies have analyzed the association between eNOS polymorphisms and CAD [1, 8]. Another molecule, Apolipoprotein E (ApoE), plays a major role in lipoprotein metabolism and the ApoE gene is polymorphic with three common alleles (ε2, ε3 and ε4), which produce three homozygous (ε2/2, ε3/3 and ε4/4) and three heterozygous (ε2/3, ε2/4 and ε3/4) genotypes [2, 9]

We previously evaluated the impact of ACE I/D polymorphism, eNOS intron 4 a/b variable number of tandem repeats (VNTR) polymorphism and ApoE genotypes on CAD[1–3]. This retrospective study investigates the relationship between genetic

polymorphisms and the severity of CAD and evaluates their potential interactions.

Material and Methods

Patients

The study population consisted of 239 patients (161 male and 78 female, with a mean age of 54.1 ± 10.3 years) who were admitted for diagnostic coronary angiography for the assessment of a suspected or confirmed clinical diagnosis of CAD. As a routine procedure, an informed written consent was obtained from all patients before angiography. The study was also approved by the local Ethics Committee.

Risk Factor Assessment

The risk factors were considered as hypertension (HT), hyperlipidemia, diabetes mellitus (DM), cigarette smoking and family history of CAD. A sustained blood pressure greater than 140 mm Hg systolic and 90 mmHg diastolic or using an antihypertensive medication was defined as HT [10]. DM was defined as hyperglycemia, requiring antidiabetic drugs or testing blood sugar over 6.7 mmol/L [11]. Patients reporting cigarette use during the year prior to examination were considered as smokers. Hyperlipidemia was defined as plasma low-density lipoprotein cholesterol (LDL-C) > 3.37 mmol/L or using lipid-lowering drugs at the time of investigation, hypertriglyceridemia was defined as triglyceride (TG) level > 1.70 mmol/L and high-density lipoprotein cholesterol (HDL-C) was considered present if the concentration of HDL-C was < 1.04 mmol/L according to The Third Report of The National Cholesterol Education Program (NCEP ATP III) guidelines [12]. NonHDL-C was defined as the difference between total cholesterol (TC) and HDL-C.

Coronary Angiography

Coronary angiography was performed according to the Judkins technique and images of the coronary tree were obtained in routine, standardized projections. The angiograms were assessed by at least two cardiologists. The coronary tree was divided into 15 segments and each segment was graded on a 4-level scale as conventionally used [13, 14]. Patients without angiographic lesions were considered as patients without CAD. Coronary artery diameter narrowing was determined according to the previously described method as 0, no visible wall irregularity or stenosis ≤ 25%; 1, stenosis ≤ 50%; 2, stenosis ≤ 75%; 3, stenosis ≥ 75 [13]. CS was defined as the number of coronary arteries (0–3) with a stenosis ≥ 75 % [15]. Severity score was calculated as the average grade of the coronary segments graded 1 or more [14].

Laboratory Analysis

Blood Chemistry

Venous blood samples were collected after a 12-h fasting period. Plasma was separated within 4 h and stored at – 20°C. Subsequent analysis of plasma TC and TG was performed using enzymatic and colorimetric assays (CHOD-PAP and GPO-PAP methods, respectively). Plasma HDL-C was measured using an enzymatic and colorimetric method (CHOD-PAP) without sample pretreatment. Plasma LDL-C was calculated using the Friedewald formula when the TG levels were < 4.5 mmol/L. In case of high levels of TG (> 4.5 mmol/L), a direct enzymatic and colorimetric method (CHOD-PAP) without sample pretreatment was used. Non-HDL cholesterol was defined as the difference between TC and HDL-C [12].

Genetic Analysis

Venous blood samples were collected into EDTA. Genomic DNA from leukocytes was purified according to the method of Miller [16].

Determination of ACE I/D Polymorphism

A 287 bp I/D polymorphism in intron 16 of the ACE gene was examined with PCR by using the primer sequences of the targeted region of the genome (17q23) [17].

The template DNA (0.4 mg per sample) was amplified by the following primers: (forward) 5'-CTG GAG ACC ACT CCC ATC CTT TCT 3', and (reverse) 5'-GAT GTG GCC ATC ACA TTC GTC AGA T 3'. These primers (10 pmol of each) were added to a mixture containing 5 μL of 10X Cetus buffer (pH 8.3), 0.5 mM dNTP (dATP, dCTP, dGTP, dTTP) and 1.0 units of Taq DNA polymerase (Perkin Elmer Cetus). A PCR program (Perkin Elmer 9600 Thermal Cycler) was initiated in a final total of 50 mL volume with thirty cycles of denaturation for 1 min at 94°C, annealing for 1 min at 58°C and primer extension for 1 min at 72°C was applied for amplification. PCR products of ACE gene locus were examined by agarose gel electrophoresis (3% agarose) at 150 V for 60 min and visualized at room temperature under UV after ethidium bromide staining (Fig. 1).

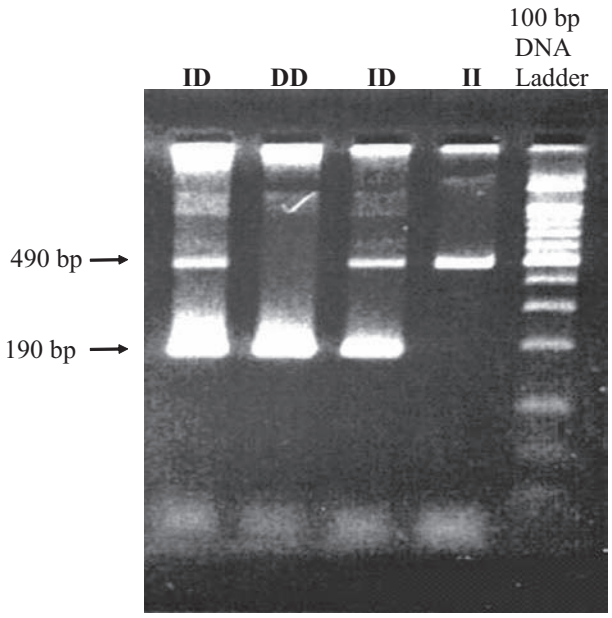

Fig. 1. Polymorphism in intron 16 of the ACE gene. The PCR products were examined by agarose gel electrophoresis (3% agarose) at 150 V for 60 min and visualized at room temperature under UV after ethidium bromide staining

Determination of eNOS 4 a/b VNTR Polymorphism

The eNOS gene locus, located on chromosome 7q 35 to 36, which comprises 26 exons spanning 21 kilobases, shows a 27-bp repeat polymorphism in intron 4 of the eNOS gene [intron 4VNTR]. The eNOS gene has two common alleles containing 4 repeats (a) and 5 repeats (b) which produce two homozygous (aa and bb) and one heterozygous (ab) genotype [1].

eNOS gene intron 4, 27 bp. VNTR polymorphism was detected by PCR according to the method described by Wang et al. [18]. The template DNA (0.5 μg per sample) was amplified using the following primers: (forward) 5'-'AGG CCC TAT GGT AGT GCC TTT-3' and 5'-TCT CTT AGT GCT GTG CTC AC-3' (reverse). These primers (25 pmol of each) were added to a mixture containing 0.2 μmol/L each of the dATP, dCTP, dGTP, dTTP, 5 μL of 10x Cetus buffer (pH 8.3), 5 μL of DMSO (100%) and 0.5 units of Taq DNA polymerase (Perkin Elmer Cetus) in a final volume of 50 μL. The PCR was initiated with a denaturation by first heating the samples for 5 min at 94°C. Thirty five cycles of denaturation for 1 min at 94°C, annealing for 1 min at 56°C, primer extension for 2 min at 72°C and last extension for 5 min at 72°C was applied for amplification. PCR products of NO gene locus were examined by gel electrophoresis (2 % NuSieve agarose-agarose) at 150 V for 30 min and visualized at room temperature under UV after ethidium bromide staining (Fig. 2).

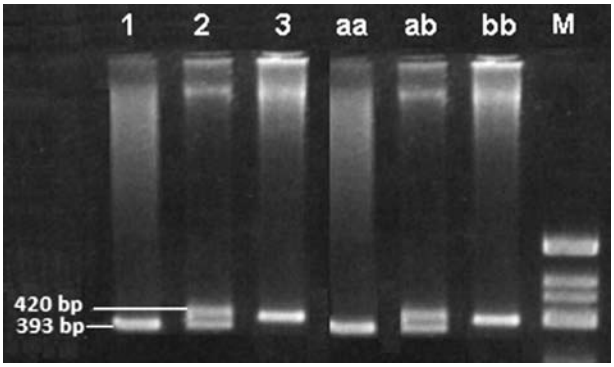

Fig. 2. Polymorphism in intron 4 of the eNOS gene. The PCR products were examined by gel electrophoresis (2%NuSieve agarose–agarose) at 150 V for 30 minutes and visualized at room temperature under UV after ethidium bromide staining, 1 – aa control, 2 – ab control, 3 – bb control, M – Marker (x174 DNA*Hinf* I)

Determination of ApoE Genotypes

The ApoE gene locus located on chromosome 19 shows a polymorphism with three common alleles (ε2, ε3 and ε4), which produce three homozygous (ε2⁄2, ε3⁄3 and ε4⁄4) and three heterozygous (ε2⁄3, ε2⁄4 and ε3⁄4) genotypes [2, 19].

ApoE gene polymorphism was detected by PCR according to the method described by Wenham et al. [19]. The template DNA (0.4 μg per sample) was amplified using the following primers: (forward) 5'-TCCAAGGAGCTGCAGCTGCAGGCGGCGCA-3' and (reverse) 5'-ACAGAATTCGCCCCGGC-3'. These primers (25 pmoL of each) were added to a mixture containing 0.2 μmoL/each of the dATP, dCTP, dGTP, DTTP, 5 μL of 10X Cetus buffer (pH 8.3), 10 μL of DMSO (50%) and 1.5 units of Taq DNA polymerase (Perkin Elmer Cetus) in a final volume of 50 μL. The PCR was initiated with a denaturation by first heating the samples for 5 min at 95°C. Forty cycles of denaturation for 0.5 min at 94°C, annealing for 0.5 min at 65°C and primer extension for 1.5 min at 70°C were applied for amplification. PCR products of ApoE locus were digested with a CfoI restriction enzyme. Then the samples were examined by gel electrophoresis (3% agarose) at 150 V for 30 min after incubation at 37°C overnight and visualized at room temperature under UV after ethidium bromide staining (Fig. 3).

Statistical Analysis

All statistical analyses were performed using the SPSS v15.0 package program. Categorical data such as sex, hypertension, etc. were presented as

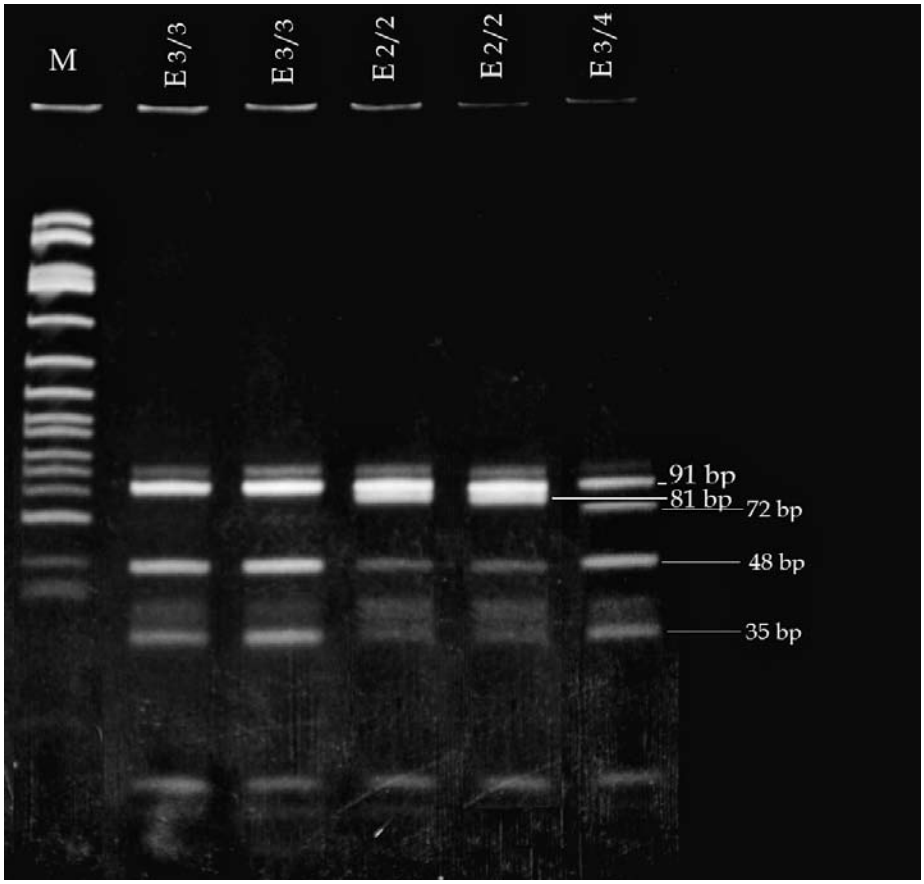

Fig. 3. The apoE gene locus located on chromosome 19 shows a polymorphism with three common alleles (ε2, ε3 and ε4). PCR products of Apo E locus were digested with CfoI restriction enzyme. Then, the samples were examined by gel electrophoresis (3% agarose) at 150 V for 30 min after the incubation at 37°C overnight and visualized at room temperature under UV after ethidium bromide staining, M – Marker, ε2/2 – 91bp–81bp, ε2/3 – 91bp–81bp–48bp–33bp, ε2/4 – 91bp–81bp–72bp–48bp–33bp, ε3/3 – 91bp–48bp–33bp, ε3/4 – 91bp–72bp–48bp–33bp, ε4/4 – 72bp–48bp–33bp.

percents and, for continuous data, mean and standard deviation were used. To evaluate gene-gene interaction, all possible subgroups were created. A Chi square test was used to evaluate the trend in severity score and CS. ANOVA (or Kruskal Wallis Test) was applied to compare age and lipid measurements in severity score and CS groups. In all analyses, *p* values less than 0.05 were considered statistically significant.

Results

Demographic patterns and the gene distributions of the patients are shown in Table 1.

Coronary and Severity Scores and Risk Factors

CS is significantly higher in male patients ($p < 0.001$), patients with DM ($p = 0.003$), and smokers ($p = 0.006$). It is also higher in patients with HT, although the difference is not significant ($p = 0.052$) (Table 2). SS is also higher in males ($p < 0.001$), diabetics ($p = 0.006$) and smokers ($p < 0.001$) (Table 3). There was no relationship between CS and SS and lipid profile (Tables 2 and 3, respectively).

Risk Factors and Genes

In patients with HT, the coexistence of ACE DD allele increases the SS ($p = 0.024$). This is not observed in patients without HT ($p = 0.323$).

Coronary and Severity Scores and Genes

Gene distributions according to the CS and the SS are shown in Tables 2 and 3. Coronary and severity scores did not show a direct relationship with eNOS and ApoE genotypes. There is also no relationship between ACE and CS ($p = 0.095$) (Table 2). However, in patients carrying the ACE DD allele, the severity score was higher than the patients with other ACE alleles ($p = 0.031$) (Table 3). When gene interactions were investigated, SS were higher only in patients with coexisting eNOS b/b genotype and ACE DD allele ($p = 0.043$) (Table 4).

Discussion

The present study investigated the effects of gene-gene interaction on CAD severity and showed that only ACE I/D polymorphism was associated with CAD severity, whereas eNOS 4a/b VNTR

Table 1. Demographic patterns and the gene distributions of the patients

	n = 239
Age (years, mean ± S.D.)	54.1 ± 10.3
Gender (M/F)	161/78
HT (n, %)	95 (39.7)
DM (n, %)	33 (13.8)
Cigarette smoking (n, %)	97 (40.6)
HDL – C (mmol/L)	1.10 ± 0.25
LDL – C (mmol/L)	3.27 ± 0.97
TG (mmol/L)	2.01 ± 1.16
TC (mmol/L)	5.32 ± 1.15
Non-HDL – C (mmol/L)	4.21 ± 1.13
Apo E Genotypes	n, (%)
ε 2/2	23 (9.6)
ε 3/2	7 (2.9)
ε 3/3	179 (74.9)
ε 4/3	28 (11.7)
ε 4/4	2 (0.8)
eNOS Genotypes	n, (%)
a/a	11 (4.6)
a/b	59 (24.7)
b/b	169 (70.7)
ACE Genotypes	n, (%)
DD	111 (46.4)
ID	118 (49.4)
II	10 (4.2)

ACE – Angiotensin converting enzyme, DM – diabetes mellitus, eNOS – Endothelial nitric oxide synthase, F – female, HDL-C – high density lipoprotein cholesterol, HT – hypertension, M–male, LDL-C – low-density lipoprotein cholesterol, TC – total cholesterol, TG – triglyceride.

polymorphism and ApoE genotypes were not associated with severity of CAD. We previously found that ApoE polymorphism (presence of ε4 allele), I/D polymorphism of the ACE gene (carrying D allele) and eNOS intron a/b polymorphism (presence of *a* allele) were the risk factors for CAD in Southern Turkey [1–3]. These findings were consistent with other studies [4, 5, 20–23]. We did not evaluate whether these individual genes have an effect on the severity of CAD.

It is known that serum lipid levels are strongly correlated with atherosclerosis [24]. Lipid accumulation may lead to a cascade of events resulting in serious obstructive atherosclerotic lesions. It is hard to determine the hemodynamic consequences of obstructions with angiography, since quantitative angiography is not sufficient for 3-dimensional sensitive evaluation of coronary lesions. However, clinical symptoms in atherosclerosis usually evolve due to degeneration and rupture of the atherosclerotic plaque, rather than its stable development. This may be an explanation as to why the severity of coronary artery obstruction might not be associated with clinical disease severity.

In addition to conventional risk factors, genetics also contributes to CAD development. A great number of genetic polymorphisms may be involved in the development of CAD. On the other hand, interactions of multiple genes may also play a role in the development of CAD. Genetic polymorphisms and interactions of different genes on CAD have been subject to many studies in medicine [23, 25]. The association of ApoE and ACE genotypes with healthy aging have been investigated, and a significantly higher frequency of ApoE/ε2 was observed in men between 60–90 years of age. No relationship has been observed in ApoE and ACE gene polimorphisms [25]. Genetic polymorphisms have also been studied in other cardiovascular diseases. XuGung et al. [26] found unfavorable genotype combinations, which act synergistically in the development of ischemic stroke. In a study, ACE I/D polymorphism was found to be positively associated with type 2 DM and synergistic effects of DD-33 and ID-23 were also shown. No association was found with ApoE polymorphism [27].

The CORGENE study [14] investigated the relationship between renin-angiotensin system genetic polymorphisms and the severity and/or extent of coronary atherosclerosis, evaluated their potential interactions and no significant associations were found.

There is conflicting data on gene-gene interactions. In some studies, significant interactions between ApoE and ACE alleles have been found in patients with stroke and Alzheimer's disease [28, 29]. A significant interaction was found between PPARγ CT and ApoE/ε4 genotypes in patients with CAD [30]. eNOS and ACE gene polymorphisms were examined in patients with CAD and eNOS gene polymorphism was found as a frequent risk factor for vascular abnormalities in CAD [20]. Ji et al. [21] found an association between ACE and eNOS gene polymorphisms and CAD risk. However, in this study, the diagnosis of CAD was not based on coronary angiography. Another study showed that ACE gene polymorphism was a significant predictor for CAD, but was not a marker for the severity of coronary atherosclerosis [22]. On the other hand Qiu et al. [31] showed that ACE (DD genotype) and angiotensin II type 1 receptor gene (C allele) polymorphism increased the risk of CAD.

In our study, coronary and severity scores did not show a relationship with eNOS and ApoE genotypes. There was also no relationship between ACE and CS ($p = 0.095$) (Table 2). However, in patients carrying the ACE DD allele, the

Table 2. Gene distributions according to the coronary score

Variables	Coronary Score (CS)				P
	0	1	2	3	
n	127	59	41	12	
Age (Mean ± SD)	53.92 ± 10.41	53.41 ± 9.89	54.05 ± 10.08	58.67 ± 10.81	0.446
Gender (M/F)	67/60	54/5	31/10	9/3	< 0.001
ACE (DD/ID/II)	51/68/8	31/26/2	24/17/0	5/7/0	0.034
eNOS (aa/ab/bb)	5/30/92	2/15/42	3/11/27	1/3/8	0.314
Apo E 2/2 2/3 3/3 3/4 4/4	 14 4 94 13 2	 6 1 42 10 0	 3 2 33 3 0	 0 0 10 2 0	0.419
HT (%)	46 (36.5)	22 (37.9)	19 (46.3)	8 (66.7)	0.052
DM (%)	11(8.7)	9 (15.5)	9 (22.0)	4 (33.3)	0.003
Smoking (%)	37 (29.4)	35 (60.3)	19 (46.3)	6 (50.0)	0.006
TG (mmol/L)	1.97 ± 1.02	1.84 ± 1.13	2.41 ± 1.47	1.85 ± 1.18	0.091
TC (mmol/L)	5.22 ± 0.96	5.31 ± 1.07	5.43 ± 1.33	6.14 ± 2.07	0.384
LDL-C (mmol/L)	3.15 ± 0.80	3.41 ± 0.86	3.19 ± 1.05	4.21 ± 1.93	0.058
HDL-C (mmol/L)	1.13 ± 0.27	1.11 ± 0.20	1.07 ± 0.22	1.02 ± 0.19	0.069
NonHDL-C (mmol/L)	4.08 ± 0.93	4.20 ± 1.10	4.36 ± 1.23	5.12 ± 2.13	0.176
Apo E 2/2 or 2/3 3/3 3/4 or 4/4	 18 94 15	 7 42 10	 5 33 3	 0 10 2	0.481
ACE (DD/not DD)	51/76	31/28	24/17	5/7	0.095
eNOS (bb/not bb)	92/35	42/17	27/14	8/4	0.424

ACE – Angiotensin converting enzyme, DM– diabetes mellitus, eNOS – Endothelial nitric oxide synthase, F – female, HDL-C – high density lipoprotein cholesterol, HT – hypertension, M – male, LDL-C – low-density lipoprotein cholesterol, TC – total cholesterol, TG – triglyceride.

severity score was higher than patients with other ACE alleles (p = 0.031) (Table 3). When gene interactions were investigated, there was an increase in the severity score of patients with coexisting eNOS b/b genotype and ACE DD allele (p = 0.043) (Table 4). Variability of the results of the different studies could be partially explained by differences in ethnicities of the studied populations, environmental factors, studied genes, methods used to determine CAD and also statistical methods.

In conclusion, the interactions of investigated gene polymorphisms do not play any important role in determining an individual's risk for the severity of CAD. Thus, genotype screening is not a useful method in the evaluation of the severity of coronary atherosclerosis in clinical practice. Using conventional risk factors is easier than using complex genetic factors for CAD risk determination in daily clinical practice.

Table 3. Gene distributions according to the severity score

Variables	Severity Score (SS)				P
	0	**1 – 1.66**	**2 – 2.66**	**3**	
n	88	23	77	51	
Age (Mean ± SD)	53.45±10.02	55.13 ± 10.92	54.03 ± 10.11	54.67±10.76	0.866
Gender (M/F)	45/43	13/10	63/14	40/11	< 0.001
ACE (DD/ID/II)	33/48/7	11/11/1	39/37/1	28/22/1	0.009
eNOS (aa/ab/bb)	1/18/69	4/5/14	4/21/52	2/15/34	0.117
Apo E 2/2 2/3 3/3 3/4 4/4	 12 2 65 8 1	 2 0 17 4 0	 8 3 54 11 1	 1 2 43 5 0	0.144
HT (%)	32 (36.4)	9 (39.1)	29 (37.7)	25 (49.0)	0.227
DM (%)	5 (5.7)	5 (21.7)	11 (14.3)	12 (23.5)	0.006
Smoking (%)	22 (25.0)	8 (34.8)	36 (46.8)	31 (60.8)	< 0.001
TG (mmol/L)	1.96 ± 1.01	1.94 ± 1.07	2.00 ± 1.24	2.13 ± 1.30	0.912
TC (mmol/L)	5.15 ± 1.01	5.38 ± 0.85	5.40 ± 1.00	5.47 ± 1.59	0.387
LDL-C (mmol/L)	3.14 ± 0.87	3.05 ± 0.59	3.31 ± 0.83	3.53 ± 1.34	0.180
HDL-C (mmol/L)	1.13 ± 0.13	1.17 ± 0.19	1.09 ± 0.17	1.08 ± 0.24	0.091
NonHDL-C (mmol/L)	4.02 ± 0.97	4.21 ± 0.87	4.31 ± 0.96	4.39 ± 1.60	0.243
Apo E 2/2 or 2/3 3/3 3/4 or 4/4	 14 65 9	 2 17 4	 11 54 12	 3 43 5	0.276
ACE (DD/not DD)	33/55	11/12	39/38	28/23	0.031
eNOS (bb/not bb)	69/19	14/9	52/25	34/17	0.109

ACE – Angiotensin converting enzyme, DM – diabetes mellitus, eNOS – endothelial nitric oxide synthase, F – female, HDL-C – high density lipoprotein cholesterol, HT–hypertension, M – male, LDL-C – low-density lipoprotein cholesterol, TC – total cholesterol, TG – triglyceride.

Table 4. Gene – gene and gene – risk factors interaction according to the severity score

	Severity Score (SS)				P
	0	**1 – 1.66**	**2 – 2.66**	**3**	
eNOS bb genotype ACE (DD/not DD)	23/46	7/7	27/25	17/17	0.043
eNOS ab genotype ACE (DD/not DD)	9/9	2/3	10/11	10/5	0.418
eNOS aa genotype ACE (DD/not DD)	1/0	2/2	2/2	1/1	0.592

ACE – Anjiotensin convertig enzym, DM – diabetes mellitus, eNOS – endothelial nitric oxide synthase, HT – hypertension.

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