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Original Research

Common Genetic Polymorphisms and Environmental Risk Factors in Polish Patients with Angiographically Documented Coronary Artery Disease

Jarosław Gorący[1], Iwona Gorący[2]*, Mariusz Kaczmarczyk[2], Mirosław Brykczyński[3], Katarzyna Widecka-Ostrowska[1], Olga Taryma[2], Andrzej Ciechanowicz[2]

[1]Department of Cardiology, Pomeranian Medical University,
[2]Department of Clinical and Molecular Biochemistry, Pomeranian Medical University,
[3]Department of Cardiosurgery, Pomeranian Medical University,
Powstańców Wlkp. 72, 70-111 Szczecin, Poland

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Abstract

It has been suggested that the G894T *NOS3*, C677T *MTHFR*, A(-455)G *FBB,* and C(-1562)T *MMP-9* genetic polymorphisms were implicated in the pathogenesis of CAD. We assessed their prevalence among CAD and controls.

A total of 180 individuals with angiographically documented CAD (138 males and 42 females, age range 37-84 years) were recruited into the study. 133 patients with ≥50% occlusion of the coronary artery lumen in angiography comprised the CAD group, a subgroup of 45 patients with one vessel occlusion as CAD_1, 88 patients with multivessel occlusion as CAD_{2+3} and the control group consisted of 47 subjects without changes in coronary arteries. Risk factors (gender, BMI, smoking, diabetes mellitus, hypertension, lipid profile) were considered for all participants. Genotype analysis was assessed by PCR-RFLP. A logistic regression analysis with CAD and CAD severity (CAD_{2+3} *vs.* CAD_1) as dependent variables was performed to estimate the age, gender, and cardiovascular risk factors (age, gender, BMI, smoking, hypertension) adjusting odds ratios for the genotypes. None of the polymorphisms studied were shown to be independently associated with an increased risk of CAD or multivessel CAD disease, in any mode of inheritance. A highly increased risk (OR 9.59) of the predisposition to advanced CAD, although only marginally significant, was observed in TT *MMP-9* homozygotes. Our results suggest a lack of association between G894T *NOS3*, A(-455)G *FBB*, C677T *MTHFR*, or C(-1562)T *MMP-9* genetic variants and CAD in Polish patients. Although a higher prevalence of classical risk factor was observed in our CAD patients.

Keywords: coronary disease, nitric oxide synthase, fibrinogen, methylene, tetrhydrofolate reductase, metalloproteinase-9, polymorphism

*e-mail: igor@sci.pam.szczecin.pl

Introduction

Coronary artery disease (CAD) is a disease with a complicated background. The role of conventional factors does not fully explain its development. This fact has led to attempts at identification of alternate determinants of risk with mechanistic rationale. Many known candidate genes have a huge influence on the development of CAD, but the mechanisms of interaction intensifying effects of these genes are still under research. Nitric oxide (NO), synthesized by endothelial nitric oxide synthase (*NOS3*) from l-arginine, is a mediator of normal endothelial function [1, 2]. As a smooth muscle relaxant, it promotes vasodilatation, is an inhibitor of smooth muscle proliferation and a platelet inhibitor, as well as limiting the oxidation of atherogenic low-density lipoproteins [3-5]. A common variant of G894T *NOS3* gene, located in exon7, causes the change of Glu298Asp in the polypeptide chain, that may alter *NOS3* activity or regulation. This polymorphism is reported to be a strong risk factor in coronary artery disease [6], coronary spasms [7], and hypertension [8]. Homocysteine (Hcy) is a recognized risk factor of coronary artery disease [9]. Methylenetetrahydrofolate reductase (*MTHFR*) is an enzyme that catalyzes the conversion of 5,10-methylenetetrahydrofolate into 5-methylenetetrahydrofolate and plays a key role in the metabolism of Hcy. Functional polymorphism of *MTHFR* C677T causes enzyme thermolability and its lower activity, which leads to hyperhomocysteinemia. It was observed that this polymorphism is correlated with an increased risk of CAD [10, 11], although simultaneous studies have been published that did not confirm this correlation [12, 13]. Fibrinogen is an acute phase protein. Elevated levels of fibrinogen may reflect inflammation of the vascular wall. The effect of fibrinogen on the development of atherosclerosis takes place mostly through promotion of platelet adhesion and leukocytes to the endothelial surfaces [14, 15]. Many studies reported a strong correlation between elevated plasma levels of fibrinogen and an increased risk of myocardial infarction, stroke, and thrombotic risk with venous thrombosis [16-18]. The three chains of fibrinogen are encoded by different genes [19, 20]. Polymorphism of the β-fibrinogen (*FBB*) gene A(-455)G is associated with differences in the plasma levels of fibrinogen. In patients who have the -455A allele, the level of fibrinogen is higher, while in heterozygotes it is average [19, 21]. This finding suggests that patients with rare -455A allele are more prone to prevalence/development of artery disease [21].

The matrix metalloproteinase-9 is known as gelatinase B, is classified as a zinc-dependent enzyme, and demonstrates proteolytic activity mostly against collagen proteoglycans and elastin. High activity of this enzyme occurs in various pathological conditions, especially in inflammation, tumor metastases, aneurysms, and myocardial injury [23, 24]. The role of metalloproteinase-9 in the pathogenesis of atherosclerosis is compound. The role the enzyme plays in the destabilization of atherosclerotic plaque seems important, particularly the observed increased activity in the vulnerable regions of atherosclerotic plaques. It has been suggested that metalloproteinase-9 is causally involved in the pathogenesis of cardiovascular disease [25]. Zhang et al. described the functional polymorphism of the C(-1562) T *MMP-9* gene demonstrating that T allele has a higher enzyme activity [26, 27], but recent results in Iranian patients indicate that up-regulation of MMP activity, including *MMP-9*, is common in the falling myocardium and missing up-regulation of transcription indicates that protein levels of MMPs were regulated at the post transcriptional level [28]. Despite extensive studies that confirm the influence of genetic factors on the development of CAD, many programs that did not confirm that impact were also reported [29]. These reports are still controversial and provoke scientists to continuous research for factors of CAD development. We were looking for a synergistic relation of G984T polymorphisms of *NOS3* gene, C677T of *MTHFR* gene, G(-455)A of gene *FBB* and (-1562), of *MMP-9* gene with the development and progression of CAD in patients with angiographically confirmed coronary artery disease.

Material and Methods

The Study Group

Protocol of the study was approved by the Local Ethics Committee, with formal informed consent signed by all participants.

The patients were randomly recruited from the inpatient Clinic of Cardiology, Pomeranian Medical University, Szczecin, Poland, with 180 individuals presenting with a history of CAD and stenocardiac symptoms screened for this study. Of these, 133 patients with ≥50% occlusion of the coronary artery lumen in angiography comprised the CAD group. Of the CAD group we established a subgroup of 45 patients with one vessel occlusion as CAD_1, 88 patients with multivessel occlusion as CAD_{2+3}, and the control group consisted of 47 subjects without changes in coronary arteries. Age range of the enrolled subjects was 36-84 years for the entire group (mean 56.5±9.2), male (73.2%, age range 36-77 years, mean 55.9±8.9), and women (age range 37-84 years, mean 57.9±9.9). From the study we excluded patients with a history of myocardial infarction diagnosed according to recommendations of the Joint European Society of Cardiology/American College of Cardiology Committee [30]. Some patients were excluded from the study with clinical diagnosis of: cardiomyopathy, coagulopathy, collegenosis, and chronic inflammatory disease. Full medical history, including arterial hypertension (defined as systolic blood pressure exceeding 140 mmHg), and/or diastolic blood pressure greater than 90 mmHg, or a reported history data showing hypertension. Body mass index (BMI) was calculated as weight/height^2 and obesity was defined as higher than 25 kg/m^2. Patients were classified as "current smokers" if they reported a daily rate of more than five cigarettes.

Laboratory data on the lipid profile, serum total cholesterol (CH), triglyceride (TG), HDL, and LDL levels were

recorded with classical coronary angiography performed in all subjects. The levels of CH, TG, HDL, and LDL were measured using enzymatic methods (commercial Kit Roche Diagnostic, Poland).

Coronary angiography was performed according to standard procedures using Philips INTEGRIS HM 3000 (Philips, Netherlands) and Philips ALURA (Philips, Netherlands) devices.

Genotyping

Genomic DNA was extracted from peripheral blood leukocytes with the QIAamp DNA Mini Kit (Quiagen, Hilden, Germany). Candidate gene polymorphisms were selected based on previous association studies. The G(-455)A *FBB*, C677T *MTHFR*, G894T *NOS3* polymorphisms and C(-1562)T *MMP-9* were analyzed by PCR and restriction fragment length polymorphism (RFLP) analysis. We used primers pairs for The G(-455)A β-fibrinogen forward 5'-AAGAATTTGGGAATGCAATCTCTGCTACCT-3', reverse 5'-CTCC TCATTGTCGTTGACACCTTGGGAC-3' for C677T *MTHFR* forward 5'-CAAAGGCCAC CCC-GAAGC-3', reverse 5'-AGGACGGTGCGGTGAGAGTG-3' for G894T eNOS 5'-AA GGCAGGAGACAGTGGATG-GA-3', reverse 5'-CCCAGTCAATCCCTTTGGTGCTCA-3' and for C(-1562)T *MMP-9* forward 5'-GCCTGGCA-CATAGTAGGCCC-3' reverse 5'-CTTCCTAGCCAGC-CGGCATC-3' (TIB MOL BIOL, Poznań, Poland). PCR amplifications were performed in volumes of 20 µl containing 40 ng genomic DNA, 0.20 µl of each primers, and 10 µl 2xPCR Master Mix (Fermentas, Vilnius, Lithuania). All thermocycling was performed with 35 cycles of denaturation at 94ºC for 30 s, and annealing at 58ºC for β-fib and 64ºC for *MTHFR*, 56ºC for *NOS3* and 62ºC for *MMP-9*, uless otherwise specified, for 30 s, and extension at 72ºC for 45 s, using a Mastercycler gradient device (Eppendorf, Germany). The resulting product of *FBB* 1300 base pair (bp) length was digested with the restriction enzyme *HaeIII* (MBI Fermentas, Vilnius, Lithuania) for the (-455)G allele restriction fragments of of 834 bp, 273 bp, and 194 bp were obtained, while for the (-455)A allele restriction fragments of 1300 bp, 194 bp. The PCR product of *MTHFR* 246 bp length was digested with the restriction enzyme *HaeIII* (MBI Fermentas, Vilnius, Lithuania) for the 677T allele restriction fragments of 175 bp and 71 bp were obtained, while for the 677C allele the product remained undigested, the PCR product of *NOS3* 298 bp length was digested with the restriction enzyme *MboI* (MBI Fermentas, Vilnius, Lithuania) for the 894T allele restriction, fragments of 158 bp and 90 bp were obtained, while for the 894G allele the product remained undigested and the PCR product of *MMP-9* 435 bp length was digested with the restriction enzyme PaeI (MBI Fermentas, Vilnius, Lithuania) for the (-1562)T allele restriction, fragments of 247 and 188 bp were obtained, while for the (-1562)C allele the product remained undigested. The digestion products were separated in 3% agarose gels, stained with ethidium bromide and recorded with a DS-34 Polaroid Instant Camera (Polaroid, Germany) under UV light (Transiluminator 4000, Stratagene).

Statistical Analysis

Hardy-Weinberg equilibrium was evaluated for each polymorphism using Fisher's exact test. First, genotype and allele frequencies between groups were compared by Chi-square test. For each polymorphism, genotype frequencies between groups were then compared by logistic regression analyses adjusted for age, gender, BMI, smoking, and hypertension in order to calculate odds ratios (ORs) and 95% confidence intervals (CIs) assuming different inheritance patterns: dominant, recessive, and additive (a univariable logistic regression). Next, we performed multivariable logistic regression analyses taking all four polymorphisms (no criterion for entry was used) and we controlled for the same factors as in the univariable analyses. Clinical and biochemical parameters were compared using either Student's t-test or Chi-square/Fisher's exact tests.

Results

A total of 180 individuals (138 males and 42 females, age range 37-84 years) were recruited into the study. Of these, 133 patients (109 males and 24 females, age range 38-84 years) had confirmed CAD and 47 (29 males and 18 females, age range 37-72 years) were classified as healthy (controls). The 88 patients (74 males and 14 females, age range 41-84 years) had multivessel disease (CAD_{2+3}), 45 patients (35 males and 10 females, age range 38-67 years) had single-vessel disease (CAD_1).

Complete clinical history, including cardiovascular risk factors (gender, BMI, smoking, diabetes mellitus, hypertension, lipid profile), was taken for all participants (Table 1). First, we analyzed the genotypes and alleles distribution of A(-455)G *FBB*, G894T *NOS3*, C677T *MTHFR*, and C(-1562)T *MMP-9* genes polymorphisms among patients with CAD and healthy individuals. There was no significant difference in allelic frequency and genotype between CAD and non-CAD patients (Table 1). None of the 47 healthy patients exhibited homozygous AA of A(-455)G *FBB* and TT of C(-1562)T *MMP-9* genotypes. Similarly, the genotypes and allele distribution in patients with single-vessel disease (CAD_1) was not significantly different from patients having multivessel disease (CAD_{2+3}).

A logistic regression analysis with CAD status (CAD *vs*. Controls) and CAD severity status (CAD_{2+3} *vs*. CAD_1) as dependent variables was performed to estimate the age, gender and cardiovascular risk factors (BMI, smoking, hypertension), adjusted odds ratios for the genotypes under dominant (D), recessive (R), and additive (A) modes of inheritance (Tables 2 and 3). None of the polymorphisms studied were shown to be independently associated with an increased risk of CAD or multivessel CAD disease, in any mode of inheritance. A highly increased risk (OR 9.59) of being predisposed to advanced CAD, although only marginally significant (p=0.062) and not precisely estimated (95%CI 0.86-105.09), was observed in individuals possessing the TT of C(-1562)T *MMP-9* genotype, as compared to those having the CC and CT C(-1562)T *MMP-9* genotypes.

Table 1. Clinical, biochemical, and genetic characteristics of patients stratified by CAD status and CAD severity status.

	Controls (n=47)	CAD (n=133)	p CAD *vs*. Controls	CAD_{2+3} (n=88)	CAD_1 (n=45)	p CAD_{2+3} *vs*. CAD_1
Gender (Male/Female)	29/18	109/24	0.005	74/14	35/10	0.37
BMI (kg/m^2)	25.2±3.9	27.3±3.3	0.001	27.3±3.4	27.3±3.2	0.999
Smoking status (Yes/No)	12/35	50/83	0.134	34/54	16/29	0.729
Diabetes (Yes/No)	1/46	18/115	0.029	17/71	1/44	0.006
Hypertension (Yes/No)	15/32	82/51	0.001	57/31	25/20	0.301
Triglycerides (mg/dl)	184±120	167±67	0.243	166±65	169±72	0.804
Cholesterol (mg/dl)	226±41	223±38	0.577	222±39	224±34	0.765
HDL (mg/dl)	44±11	42±8	0.260	42±9	43±8	0.426
LDL (mg/dl)	136±33	137±33	0.902	137±35	138±30	0.856
FBB genotype (GG/GA/AA)	27/20/0	68/53/12	0.102	47/35/6	21/18/6	0.435
FBB allele Alleles (G/A)	74/20	189/77	0.150	129/47	60/30	0.259
NOS3 genotype (GG/GT/TT)	22/20/5	59/64/10	0.711	41/42/5	18/22/5	0.480
NOS3 Alleles (G/T)	64/30	182/84	0.952	124/52	58/32	0.318
MTHFR genotype (CC/CT/TT)	22/23/2	67/53/13	0.361	43/35/10	24/18/3	0.674
MTHFR Alleles (C/T)	67/27	187/79	0.858	121/55	66/24	0.439
MMP-9 genotype (CC/CT/TT)	30/17/0	86/43/4	0.456	57/30/1	29/13/3	0.194
MMP-9 Aleles (C/T)	77/17	215/51	0.818	144/32	71/19	0.566

Although none of the polymorphisms showed a significant association in the univariate factor adjusted analyses for two dependent variables (CAD and CAD_{2+3}), we decided to perform a multivariable analysis for the same dependent variables with all four polymorphisms included, assuming additive mode of inheritance and with adjustment for age, gender, and cardiovascular risk factors (BMI, smoking, hypertension) (Table 4). Using the multivariable factor adjusted analysis, we failed to indicate any of the polymorphisms studied to be independent predictors of increased risk of CAD or multivessel CAD disease.

Discussion

The pathogenesis of CAD is complex, insofar as A(-455)G *FBB*, G894T *NOS3*, C677T *MTHFR*, C(-1562)T *MMP-9* single point mutations were found to be involved in the pathogenesis of atheromatosis. Activity of some genes may regulate or modulate the maintenance of balance of coagulable state and the endothelium continuity of the vascular wall. We assessed their prevalence among CAD patients and the control group. There was no difference in the genotypes and allele frequency of A(-455)G *FBB*, G894T *NOS3*, C677T *MTHFR*, C(-1562)T *MMP-9* between CAD and control patients. ORs for, effects of G894T *NOS3*, C677T *MTHFR* and C(-1562)T *MMP-9* polymorphisms were very similar, but polymorphisms of A(-455)G *FBB* were lower and not significant. However, their contribution to the development of coronary artery disease remains controversial. In contrast to our study, other researchers have reported participation of *NOS3* G894T, A(-455) G *FBB*, C677T *MTHFR*, C(-1562) T *MMP-9* [31-35] in prevalence of CAD. Similar to our studies, there was no significant association between CAD and G894T *NOS3* polymorphism, as in the Korean population, tough polymorphism C-786T *NOS3* is associated with CAD with adjustments for cardivascular risk factors [36]. These results suggest that the genetic component of CAD is based on small to moderate effects of many genes. Associations between functional G894T *NOS3* gene polymorphism and NO synthesis have been described [6, 37]. In the Li et al. meta-analysis the synthesis of available evidence supports the fact that *NOS3* G894 T and T-786C are associated with CAD in the non-Asian population, but in this study only genetic factors are considered [38], but NO mechanism synthesis is also influenced by environmental factors such as smoking, exercise, and hypertension [39, 40]. Similarly, a functional polymorphism of A(-455)G *FBB* gene regulates fibrinogen level, which, however, may be modified by a lifestyle [41]. In the studied group low physical activity and unhealthy diet resulting in obesity may play a signifi-

Table 2. Estimated effects for *FBB*, *NOS3*, *MTHFR*, and *MMP-9* genetic polymorphisms and environmental risk factors in univariable logistic regression of CAD.

CAD *vs*. Controls												
	FBB			*NOS3*			*MTHFR*			*MMP-9*		
	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
Age	D1.04	1.00-1.08	0.058	1.04	1.00-1.08	0.057	1.04	1.00-1.09	0.054	1.04	1.00-1.09	0.055
	R -	-	-	1.04	1.00-1.08	0.057	1.04	1.00-1.09	0.052	-	-	-
	A1.04	1.00-1.08	0.063	1.04	1.00-1.08	0.058	1.04	1.00-1.08	0.057	1.04	1.00-1.09	0.055
Gender (Male/Female)	D2.38	1.00-5.67	0.049	2.15	0.93-4.96	0.072	2.16	0.93-4.98	0.070	2.17	0.94-5.01	0.069
	R -	-	-	2.14	0.93-4.95	0.073	2.13	0.92-4.94	0.075	-	-	-
	A2.56	1.07-6.13	0.034	2.14	0.93-4.96	0.073	2.14	0.93-4.94	0.073	2.16	0.93-4.98	0.070
BMI	D1.15	1.02-1.29	0.018	1.14	1.02-1.28	0.021	1.14	1.02-1.28	0.022	1.15	1.02-1.29	0.019
	R -	-	-	1.15	1.02-1.29	0.020	1.16	1.03-1.30	0.014	-	-	-
	A1.15	1.02-1.29	0.017	1.14	1.02-1.28	0.021	1.15	1.02-1.29	0.019	1.15	1.02-1.29	0.019
Smoking (Yes/No)	D1.56	0.69-3.54	0.286	1.57	0.69-3.56	0.275	1.57	0.69-3.56	0.274	1.59	0.70-3.60	0.265
	R -	-	-	1.58	0.70-3.57	0.273	1.49	0.66-3.39	0.336	-	-	-
	A1.54	0.67-3.51	0.303	1.57	0.69-3.56	0.277	1.58	0.70-3.57	0.271	1.58	0.70-3.59	0.268
Hypertension (Yes/No)	D2.37	1.08-5.18	0.029	2.52	1.14-5.57	0.021	2.50	1.15-5.45	0.020	2.44	1.12-5.32	0.024
	R -	-	-	2.45	1.13-5.33	0.023	2.51	1.15-5.49	0.020	-	-	-
	A2.35	1.07-5.14	0.032	2.51	1.15-5.50	0.020	2.46	1.13-5.34	0.023	2.46	1.13-5.36	0.023
Gene	D0.70	0.32-1.51	0.357	1.09	0.51-2.32	0.818	1.15	0.55-2.41	0.707	1.17	0.54-2.53	0.689
	R -	-	-	1.26	0.36-4.38	0.718	0.31	0.06-1.59	0.157	-	-	-
	A1.73	0.89-3.38	0.106	0.91	0.51-1.61	0.736	1.11	0.63-1.96	0.717	0.92	0.45-1.90	0.831

FBB – D: GG/GA+AA, R: GG+GA/AA, A: GG/GA/AA; *NOS3* – D: GG/GT+TT, R: GG+GT/TT, A: GG/GT/TT; *MTHFR* – D: CC/CT+TT, R: CC+CT/TT, A: CC/CT/TT; *MMP-9* – D: CC/CT+TT, R: CC+CT/TT, A: CC/CT/TT. (D – dominant, R – recessive, A – additive).

Table 3. Estimated effects for *FBB*, *NOS3*, *MTHFR*, and *MMP-9* genetic polymorphisms and environmental risk factors in univariable logistic regression of multivessel CAD (CAD_{2+3}).

CAD_{2+3} *vs.* CAD_1												
	FBB			*NOS3*			*MTHFR*			*MMP-9*		
	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p	OR	95%CI	p
Age	D1.05	1.00-1.10	0.042	1.05	1.00-1.10	0.038	1.05	1.00-1.10	0.046	1.05	1.00-1.10	0.042
	R1.05	1.00-1.10	0.037	1.05	1.00-1.10	0.041	1.05	1.00-1.10	0.041	1.05	1.01-1.10	0.027
	A1.05	1.00-1.10	0.040	1.05	1.00-1.10	0.037	1.05	1.00-1.09	0.048	1.05	1.00-1.10	0.042
Gender (Male/Female)	D1.42	0.54-3.76	0.474	1.55	0.58-4.11	0.378	1.48	0.56-3.89	0.424	1.48	0.56-3.90	0.423
	R1.44	0.54-3.81	0.460	1.48	0.56-3.92	0.426	1.41	0.53-3.74	0.484	1.43	0.53-3.82	0.474
	A1.40	0.53-3.71	0.497	1.55	0.58-4.13	0.380	1.46	0.55-3.84	0.444	1.50	0.57-3.95	0.403
BMI	D1.00	0.89-1.13	0.937	1.01	0.90-1.14	0.876	1.01	0.90-1.14	0.829	1.01	0.90-1.14	0.843
	R1.01	0.90-1.14	0.834	1.01	0.90-1.14	0.865	1.02	0.90-1.15	0.748	1.03	0.91-1.16	0.645
	A1.00	0.89-1.13	0.945	1.01	0.90-1.14	0.892	1.02	0.90-1.14	0.792	1.02	0.90-1.15	0.758
Smoking (Yes/No)	D1.27	0.57-2.84	0.556	1.20	0.54-2.67	0.659	1.24	0.56-2.75	0.601	1.23	0.55-2.75	0.602
	R1.23	0.55-2.76	0.605	1.24	0.56-2.77	0.594	1.22	0.55-2.72	0.621	1.21	0.54-2.74	0.637
	A1.27	0.57-2.85	0.553	1.20	0.54-2.68	0.655	1.23	0.55-2.74	0.607	1.25	0.56-2.78	0.586
Hypertension (Yes/No)	D1.43	0.66-3.11	0.360	1.54	0.69-3.40	0.285	1.39	0.65-3.01	0.393	1.40	0.64-3.04	0.393
	R1.39	0.64-3.01	0.403	1.44	0.66-3.13	0.355	1.45	0.67-3.15	0.345	1.55	0.71-3.40	0.271
	A1.43	0.66-3.11	0.361	1.56	0.70-3.46	0.267	1.41	0.65-3.04	0.383	1.37	0.63-2.98	0.418
Gene	D1.35	0.63-2.91	0.433	1.54	0.70-3.36	0.275	0.94	0.44-2.00	0.939	0.99	0.44-2.19	0.973
	R2.18	0.63-7.47	0.211	2.28	0.58-8.92	0.233	0.53	0.13-2.18	0.377	9.59	0.86-105.09	0.062
	A0.71	0.40-1.27	0.246	0.65	0.35-1.20	0.165	1.16	0.65-2.07	0.601	0.80	0.40-1.60	0.527

FBB – D: GG/GA+AA, R: GG+GA/AA, A: GG/GA/AA; *NOS3* – D: GG/GT+TT, R: GG+GT/TT, A: GG/GT/TT; *MTHFR* – D: CC/CT+TT, R: CC+CT/TT, A: CC/CT/TT; *MMP-9* – D: CC/CT+TT, R: CC+CT/TT, A: CC/CT/TT. (D – dominant, R – recessive, A – additive).

Table 4. Estimated effects for *FBB*, *NOS3*, *MTHFR*, and *MMP-9* in multivariate logistic regression analysis of CAD and CAD severity, factor adjusted.

	CAD *vs*. Controls			CAD_{2+3} *vs*. CAD_1		
	OR	95%CI	p	OR	95%CI	p
Age	1.04	1.00-1.08	0.067	1.05	1.00-1.10	0.038
Gender (Male/Female)	2.56	1.06-6.16	0.034	1.47	0.54-3.97	0.444
BMI	1.15	1.03-1.30	0.015	1.01	0.89-1.14	0.900
Smoking (Yes/No)	1.51	0.66-3.47	0.322	1.25	0.55-2.82	0.587
Hypertension (Yes/No)	2.35	1.06-5.22	0.035	1.57	0.70-3.51	0.269
FBB (GG/GA/AA)	1.78	0.91-3.49	0.090	0.74	0.41-1.33	0.311
NOS3 (GG/GT/TT)	0.89	0.50-1.59	0.703	0.66	0.35-1.24	0.196
MTHFR (CC/CT/TT)	1.17	0.65-2.09	0.600	1.06	0.59-1.93	0.837
MMP-9 (CC/CT/TT)	0.87	0.42-1.80	0.700	0.83	0.42-1.67	0.607

cant role in the development of CAD. Plasma homocysteine levels are associated with cardiovascular risk. Polymorphism of C677T *MTHFR* brought about a constitutive increase in homocysteine levels, which could be modified by combined folate and vitamin B supplements. New findings by Antoniades et al. [42] demonstrated that despite the significant effect of the polymorphism on plasma Hcy levels, it does not affect vascular homocysteine levels. That observation suggests that vascular endothelium is a different compartment than the circulation system [43]. In our group we confirmed the presence of TT homozygotes in only 4 CAD patients and no one from the control group. This observation suggests that this mutation in the study group does not play an important role in CAD development due to its rarity. However, we observed a slight tendency of influence of variants of gelatinase B on CAD development in the Polish population (p=0.062).This weak effect may be due to a stable form of CAD in our patients, because other researchers have reported increased activity of *MMP-9* in patients with unstable angina [44]. Recent results published by Ghaderian et al. suggest that susceptibility to acute myocardial infarction might be related to the *MMP-9* gene expression, which affects its plasma levels [45]. We have no data on fibrinogen, metalloproteinase-9, methylenetetrahydrofolate reductase, nitric oxide synthase, and plasma levels in our patients. Therefore, we also assessed the level of interaction between G894T *NOS3*, A(-455)G *FBB*, C677T *MTHFR*, C(-1562)T *MMP-9* and environmental factors such as age, gender, BMI, smoking and arterial hypertension that contribute to coronary vessel lesions. We found no significant interactions in multivariate logistic regression analysis between CAD and four studied genes. This may mean that the impact of investigated genes in the case of a stable coronary heart disease is limited. However, it should be highlighted that our study group included patients with chronic disease and in advanced CAD the influence of genetic factors may be dimmed by particularly strong environmental factors. Independent strong influence of classical/conventional/traditional risk factors like gender, BMI, diabetes, and arterial hypertension on CAD development are recognized and are in harmony with previous knowledge and research. In the Polish population some deeply rooted traditional risk factors may overshadow/lessen the impact of genetic factors. The frequency of classical risk factors such as arterial hypertension, smoking, diabetes and BMI differed significantly between CAD and control patients, which confirms their influence on this group. Despite the development of public health programs aimed at lifestyle change, "classical" factors remain common in the Polish population with a possibility that combined genetic and environmental pressures exert a strong effect in promoting atherosclerotic processes. The incorporation of gene and environment interactions into association analyses may further improve the power to detect genetic effects and may contribute to the identification of important environmental effect modifiers [46]. Some studies have investigated relationships between genes and CAD. A number of them have indicated an interrelation, whereas others failed to prove any relationship between genes and CAD. It was reemphasized that CAD is a disease of complex etiology in which the impact of environment, traditional risk factors and regulatory effect of genetic factors playing a crucial role. According to the World Health Organization, significant differences are observed in the prevalence of risk factors, dynamics, and development of IHD in various populations, which represent diverse historical, cultural, social, economic, environmental, and genetic conditions [47, 48].

In summary, our results suggest the lack of association between G894T *NOS3*, A(-455)G *FBB*, C677T *MTHFR*, or C(-1562)T *MMP-9* genetic variants and CAD in Polish patients. Although a higher prevalence of classical risk factors was observed in our CAD patients.

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