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ORIGINAL ARTICLE

ASSOCIATION STUDY OF POLYMORPHIC VARIANTS IN 9P21 LOCUS AND THE MANIFESTATION OF CORONARY ARTERY DISEASE IN BULGARIANS

R. Tzveova[1], G. Naydenova[2], T. Yaneva-Sirakova[3], S. Vandeva[4], P. Atanasov[5], V. Mitev[6], R. Kaneva[6], D. Pendicheva-Duhlenska[7]

[1]Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences – Sofia, Bulgaria
[2]Department of Propaedeutics of Internal Diseases, Medical University – Pleven, Bulgaria
[3]Clinic of Cardiology, ICU, Medical Institute of Ministry of Interior – Sofia, Bulgaria
[4]Clinical Center of Endocrinology and Gerontology, Medical University – Sofia, Bulgaria
[5]National Sports Academy – Sofia, Bulgaria
[6]Molecular Medicine Center, Department of Medical Chemistry and Biochemistry, Medical Faculty, Medical University – Sofia, Bulgaria
[7]Department of Pharmacology, Faculty of Pharmacy, Medical University – Pleven, Bulgaria

Abstract. Objective: *The variant 9p21 is correlated with coronary artery disease (CAD) in multiple studies in the European population, but we lack information for the Eastern Europeans (Caucasian). We aimed at investigating the potential association of six common polymorphic variants in 9p21 locus (rs7865618, rs1537378, rs7857345, rs10757274, rs2383206, and rs10757278) with CAD in the Bulgarian population.* ***Materials and methods:*** *The current analysis included 261 patients with angiographically documented CAD (153 with myocardial infarction and 108 without myocardial infarction) and 496 population controls. Genomic DNA was isolated from peripheral venous blood. The selected polymorphic variants in 9p21 locus were genotyped by high resolution melting (HRM) analyses (Rotor Gene, Qiagen). Allelic and genotypic frequencies for studied variants were compared between cases and controls using the* χ^2 *test.* ***Results:*** *No deviation from the Hardy-Weinberg was observed for all polymorphic variants in both patient' and control' groups (p > 0.05). Polymorphic allele A for rs7865618 was found to be higher in the patient group than in the population controls (65.08% vs 58.28%). The carrier of this allele poses a 1.4-fold higher risk of myocardial infarction development than wild-type alleles` carriers (OR 1.40 (A) CI 1.04-1.70, p = 0.019), and this dependence is not related with gender. In female, an association between the allele C of rs7857345 and a 1.64-fold increased risk of myocardial infarction was observed (OR 1.64, CI95: 1.03-2.61, p = 0.03). For the other studied polymorphisms, no statistically significant association with disease risk was found. Also, our study found a positive association between rs2383206 and decreased serum triglyceride levels and with serum level of LDL cholesterol.* ***Conclusion:*** *Further studies with a larger number of cases and controls will be needed in order to evaluate the possible association between the six studied polymorphisms and CAD/MI in Bulgarians.*

Key words: *9p21, polymorphic variants, coronary artery disease, Bulgarians*

Corresponding author: *Reni Tzveova, Institute of Experimental Morphology, Pathology and Anthropology with Museum, Bulgarian Academy of Sciences, Acad. G. Bonchev Str, bl. 25, Sofia 1113, Bulgaria, e-mail: renitzveova@abv.bg*

ORCID: *0000-0001-5045-8165*

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INTRODUCTION

Coronary artery disease (CAD) is a multifactorial disorder and its manifestation depends on the complex interaction between environmental factors and hereditary predisposition. The main risk factors for CAD are dyslipidemia, arterial hypertension, smoking, obesity, diabetes mellitus, diet and others. Data on the etiology and pathophysiology of coronary artery disease are available in the scientific literature, but the impact of genetic factors remains to be studied.

The genome-wide association study (GWAS) is a modern approach identifying genetic loci associated with a predisposition to serious diseases, including CAD. The first reports of genomic studies for coronary disease and its most common complication, myocardial infarction (MI), were obtained from two research groups in year 2007. The Ottawa Heart Genomic Study consortium and deCODE simultaneously and independently identified genetic variant rs1333049 located on chromosome 9, locus 9p21 as a major genetic risk factor for myocardial ischemia [1, 2]. There were no protein-coding genes in this region, although the two were lately identified as tumor suppressor genes – *CDKN2A* and *CDKN2B* [3].

Subsequently, polymorphic variants in 9p21 locus showed a strong statistically significant association with the onset of coronary disease in a large number of studies conducted in different population groups [4-23]. There were several possible mechanisms discussed: epigenetic silencing of major cluster genes from the functional product that is responsible for reduction of hypermethylation of CDKN2A and CDKN2B [24, 25]; vascular smooth muscle cells proliferation [26]; modulation of the function of TGFβ and thus another pathway for induction of atherogenesis [27].

The 9p21 locus includes various polymorphic variants such as rs1333049, rs10757274, rs10757278, rs2383206, and rs2383207, which are in partial or complete linkage disequilibrium. The first polymorphism in this locus, which showed a significant association with cardiac ischemia, was rs1333049. A meta-analysis of 40,000 patients with coronary atherosclerosis and the corresponding number of healthy controls showed that about 25% of European descent have two copies of the risk allele (homozygous state), leading to a 1.6-fold increased risk of CAD compared to wild-type allele carriers [28].

This variant was also associated with a higher risk of carotid atherosclerosis [29], stroke [30-33], peripheral arterial disease [34-36], heart failure [37] and cardiovascular mortality [38, 39], intracranial and abdominal aortic aneurysms [40-42], coronary stenosis [43, 44] and aortic calcification [45].

The aim of this study was to investigate the potential association of six common polymorphic variants in 9p21 locus (rs7865618, rs1537378, rs7857345, rs10757274, rs2383206, and rs10757278) with CAD in an Eastern European (Caucasian) population.

METHODS

This association study included 496 population controls and 261 patients with angiographically documented coronary atherosclerosis, 153 with previous MI and 108 without MI.

The diagnostic criteria for CAD were as recommended by the current guidelines: ≥ 70% lumen stenosis in at least one blood vessel as determined by coronary angiography or ≥ 50% in the left main; percutaneous coronary angioplasty; coronary bypass or myocardial infarction (MI). The diagnosis of MI was based on the 4th definition for myocardial infarction [46, 47].

The concentrations of serum triglycerides, total cholesterol and high-density lipoprotein (HDL) were measured in all patients and part of the control subjects. Low-density lipoprotein (LDL) cholesterol was calculated according to the Friedewald equation [48]. The clinical and demographic data for all cases and controls are based on official medical records and are presented in Table 1-2. Written informed consent was obtained from all individual participants included in the study.

Genomic DNA was isolated from peripheral venous blood samples using Chemagic Magnetic Separation Module I (PerkinElmer) according to the manufacturer's protocol. The selected polymorphic variants in 9p21 locus were genotyped by high resolution melting (HRM) (Rotor Gene, Qiagen).

Statistical analysis

Summarized statistics are presented as a mean +/- standard deviation (SE) or as a percentage. A comparison of allelic and genotypic frequencies between cases and controls for all studied polymorphic variants was made using the χ^2 test. The Hardy-Weinberg equilibrium was checked in the two studied groups. The significance level is assumed to be < 0.05.

Clinical and demographic data between the studied groups were compared with the χ^2 (gender) or t-test (age and other quantitative characteristics). The Bonferroni correction has been used for numerous of tests. Plink version 1.07 and Excel 2010 were used to perform the statistical analysis.

Table 1. Clinical and demographic data of patients with CAD only and compared to these in population controls

	CAD (N = 108)	Population controls (N = 496)	P value
Age (years)	66.27 ± 8.81	36.08 ± 12.99	< 0.0001
Gender (male)	60 (55.56)	241 (48.59)	< 0.0001
BMI (kg/m2)	29.66 ± 5.72	25.66 ± 4.91	< 0.0001
Total cholesterol (mmol/l)	5.64 ± 1.04	4.99 ± 0.94	< 0.0001
Triglycerides (mmol/l)	1.32 ± 0.67	0.93 ± 0.56	< 0.0001
LDL-cholesterol (mmol/l)	4.23 ± 1.06	3.18 ± 0.88	< 0.0001
HDL-cholesterol (mmol/l)	1.22 ± 0.37	1.62 ± 0.40	< 0.0001
Systolic arterial pressure (SAP) (mm Hg)	147.86 ± 21.83	–	–
Diastolic arterial pressure (DAP) (mm Hg)	87.50 ± 13.64	–	–

Values shown are means ± standard deviation, or numbers and frequencies.
CAD: Coronary artery disease; MI: Myocardial infarction.

Table 2. Clinical and demographic data of patients with MI and compared to these in population controls

	MI (N = 153)	Population controls (N = 496)	P value
Age (years)	66.34 ± 10.39	36.08 ± 12.99	< 0.0001
Gender (male)	92 (60.53)	241 (48.59)	< 0.0001
BMI (kg/m2)	28.36 ± 4.89	25.66 ± 4.91	< 0.0001
Total cholesterol (mmol/l)	5.94 ± 0.75	4.99 ± 0.94	< 0.0001
Triglycerides (mmol/l)	2.07 ± 0.56	0.93 ± 0.56	< 0.0001
LDL- cholesterol (mmol/l)	4.14 ± 0.79	3.18 ± 0.88	< 0.0001
HDL- cholesterol (mmol/l)	1.39 ± 0.28	1.62 ± 0.40	< 0.0001
Systolic arterial pressure (SAP) (mm Hg)	135.92 ± 10.96	–	–
Diastolic arterial pressure (DAP) (mm Hg)	83.64 ± 6.60	–	–

Values shown are means ± standard deviation, or numbers and frequencies.
CAD: Coronary artery disease; MI: Myocardial infarction.

RESULTS

For the purposes of this study, an association analysis was performed. A total of 496 controls and 261 patients with angiographically documented atherosclerosis of the coronary arteries were genotyped, 153 with MI and 108 without MI. The aim of the study was to analyze the possible association between 6 polymorphic variants (rs7865618, rs1537378, rs7857345, rs10757274, rs2383206 and rs10757278) in 9p21 locus and chronic CAD or the risk for acute MI. The variants were selected on the basis of previous reports in the literature [1, 2, 49].

All selected polymorphisms were successfully genotyped in > 97% of the tested samples. The distribution of genotypes and alleles in controls and patients was presented in Tables 3-5. All established genotype frequencies in the control group and in the patient group were in equilibrium according to Hardy-Weinberg equation (p > 0.05).

The allelic and genotypic distribution was found to be in the same range as in the Western European populations (including Caucasian).

Statistically significant association was found only for rs7865618 and MI. The polymorphic allele A was found to be higher in the patient group with MI than the population controls (65.08% vs 58.28%). The carriers of this allele had a 1.4-fold higher risk of MI than the carriers of the wild-type alleles (OR 1.40 (A) CI 1.04-1.70, p = 0.019). This dependence was not related to gender.

A similar trend was observed when comparing the allelic frequencies between the population control group and the general group of patients (including both cases with MI and with stable CAD. This relationship was also not related to gender. Polymorphic allele A was found to be higher in the cases than in the population controls (64.35% vs 58.28%). Its carriers had a 1.29-fold higher risk of MI than the carriers of the wild-type alleles (OR 1.29 (A) CI 1.05-1.56). The effect of rs7865618 was more expressed in the

Table 3. Distribution of allelic and genotypic frequencies for studied polymorphic variants in the group of patients with CAD (with and without myocardial infarction) and population controls with Bulgarian origin

Locus	Polymorphism	Genotype/ allele	Model	Total			Male			Female		
				CAD (with and without MI)	Population controls	P value	CAD (with and without MI)	Population controls	P value	CAD (with and without MI)	Population controls	P value
9p21	rs7865618	GG	Genotypic	42 (12.96)	84 (16.97)	0.04	21 (10.14)	32 (13.33)	0.24	21 (17.95)	52 (20.39)	0.13
		GA		147 (45.37)	245 (49.49)		104 (50.24)	130 (54.17)		43 (36.75)	115 (45.10)	
		AA		135 (41.67)	166 (33.54)		82 (39.61)	78 (32.50)		53 (45.30)	88 (34.51)	
		G	Allelic	231 (35.65)	413 (41.72)	0.01	146 (39.02)	194 (40.42)	0.13	85 (36.32)	219 (42.94)	0.09
		A		417 (64.35)	577 (58.28)		268 (60.98)	286 (59.58)		149 (63.68)	291 (57.06)	
9p21	rs1537378	TT	Genotypic	38 (13.67)	79 (15.99)	0.36	22 (13.66)	40 (16.60)	0.45	16 (15.38)	39 (15.42)	0.37
		TC		122 (44.85)	237 (47.98)		83 (51.55)	123 (51.04)		39 (37.50)	114 (45.06)	
		CC		112 (41.18)	178 (36.03)		63 (39.13)	78 (32.36)		49 (47.12)	100 (39.52)	
		T	Allelic	198 (36.40)	395 (39.98)	0.17	127 (37.80)	203 (42.12)	0.22	71 (34.13)	192 (37.94)	0.34
		C		346 (63.60)	593 (60.02)		209 (62.20)	279 (57.88)		137 (65.87)	314 (62.06)	
9p21	rs7857345	TT	Genotypic	23 (7.14)	39 (7.89)	0.65	12 (5.85)	13 (5.42)	0.98	11 (9.40)	26 (10.24)	0.24
		TC		117 (36.34)	192 (38.87)		83 (40.49)	97 (40.42)		34 (29.06)	95 (37.40)	
		CC		182 (56.52)	263 (53.24)		110 (53.66)	130 (54.16)		72 (61.54)	133 (52.36)	
		T	Allelic	163 (25.31)	270 (27.33)	0.37	107 (26.10)	123 (25.63)	0.87	56 (23.93)	147 (28.94)	0.16
		C		481 (74.69)	718 (72.67)		303 (73.90)	357 (74.37)		178 (76.07)	361 (71.06)	
9p21	rs10757274	AA	Genotypic	55 (17.08)	103 (20.81)	0.18	36 (17.48)	57 (23.65)	0.15	19 (16.38)	46 (18.11)	0.53
		AG		161 (50.00)	256 (51.72)		106 (51.46)	125 (51.87)		55 (47.41)	131 (51.58)	
		GG		106 (32.92)	136 (27.47)		64 (31.06)	59 (24.48)		42 (36.21)	77 (30.31)	
		A	Allelic	271 (42.08)	462 (46.67)	0.07	178 (43.20)	239 (49.59)	0.06	93 (40.09)	223 (43.90)	0.33
		G		373 (57.92)	528 (53.33)		234 (56.80)	243 (50.41)		139 (59.91)	285 (56.10)	
9p21	rs2383206	AA	Genotypic	57 (19.52)	107 (21.93)	0.68	39 (21.20)	45 (18.99)	0.85	18 (16.67)	62 (24.70)	0.23
		AG		147 (50.34)	233 (47.75)		93 (50.54)	123 (51.90)		54 (50.00)	110 (43.82)	
		GG		88 (30.14)	148 (30.32)		52 (28.26)	69 (29.11)		36 (33.33)	79 (31.48)	
		A	Allelic	261 (44.69)	447 (45.80)	0.67	171 (46.47)	213 (44.94)	0.66	90 (41.67)	234 (46.61)	0.22
		G		323 (55.31)	529 (54.20)		197 (53.53)	261 (55.06)		126 (58.33)	268 (53.39)	
9p21	rs10757278	AA	Genotypic	68 (21.25)	111 (22.79)	0.85	48 (23.42)	60 (25.10)	0.92	20 (17.39)	51 (20.56)	0.72
		AG		163 (50.94)	247 (50.72)		103 (50.24)	118 (49.37)		60 (52.17)	129 (52.02)	
		GG		89 (27.81)	129 (26.49)		54 (26.34)	61(25.52)		35 (30.44)	68 (27.42)	
		A	Allelic	299 (46.72)	469 (48.15)	0.57	199 (48.54)	238 (49.79)	0.71	100 (43.48)	231 (46.57)	0.44
		G		341 (53.28)	505 (51.85)		211 (51.46)	240 (50.21)		130 (56.52)	265 (53.43)	

Table 4. Distribution of allelic and genotypic frequencies for studied polymorphic variants in the group of patients with CAD (without myocardial infarction) and population controls with Bulgarian origin

Locus	Polymorphism	Allele 1	Model	Total			Male			Female		
				CAD (without MI)	Population controls	P value	CAD (without MI)	Population controls	P value	CAD (without MI)	Population controls	P value
9p21	rs7865618	GG	Genotypic	18 (14.40)	84 (16.97)	0.31	8 (10,53)	32 (13,33)	0.50	10 (20,41)	52 (20,39)	0.48
		GA		56 (44.80)	245 (49.49)		38 (50,00)	130 (54,17)		18 (36,73)	115 (45,10)	
		AA		51 (40.80)	166 (33.54)		30 (39,47)	78 (32,50)		21 (42,86)	88 (34,51)	
		G	Allelic	92 (36.80)	413 (41.72)	0.16	54 (35,53)	194 (40,42)	0.28	38 (38,78)	219 (42,94)	0.44
		A		158 (63.20)	577 (58.28)		98 (64,47)	286 (59,58)		60 (61,22)	291 (57,06)	
9p21	rs1537378	TT	Genotypic	11 (10.00)	79 (15.99)	0.16	6 (9,09)	40 (16,60)	0.26	5 (11,36)	39 (15,42)	0.41
		TC		51 (46.36)	237 (47.98)		34 (51,52)	123 (51,04)		17 (38,64)	114 (45,06)	
		CC		48 (43.64)	178 (36.03)		26 (39,39)	78 (32,37)		22 (50,00)	100 (39,53)	
		T	Allelic	73 (33.18)	395 (39.98)	0.06	46 (34,85)	203 (42,12)	0.13	27 (30,68)	192 (37,94)	0.19
		C		147 (66.82)	593 (60.02)		86 (65,15)	279 (57,88)		61 (69,32)	314 (62,06)	
9p21	rs7857345	TT	Genotypic	11 (8.80)	39 (7.89)	0.95	5 (6,58)	13 (5,42)	0.92	6 (12,24)	26 (10,24)	0.89
		TC		48 (38.40)	192 (38.87)		31 (40,79)	97 (40,42)		17 (34,69)	95 (37,40)	
		CC		66 (52.80)	263 (53.24)		40 (52,63)	130 (54,17)		26 (53,06)	133 (52,36)	
		T	Allelic	70 (28.00)	270 (27.33)	0.83	41 (26,97)	123 (25,63)	0.74	29 (29,59)	147 (28,94)	0.9
		C		180 (72.00)	718 (72.67)		111 (73,03)	357 (74,38)		69 (70,41)	361 (71,06)	
9p21	rs10757274	AA	Genotypic	23 (18.70)	103 (20,81)	0.63	15 (20,00)	57 (23,65)	0.31	8 (16,67)	46 (18,11)	0.94
		AG		61 (49.59)	256 (51,72)		35 (46,67)	125 (51,87)		26 (54,17)	131 (51,57)	
		GG		39 (31.71)	136 (27,47)		25 (33,33)	59 (24,48)		14 (29,17)	77 (30,31)	
		A	Allelic	107 (43.50)	462 (46.67)	0.37	65 (43,33)	239 (49,59)	0.18	42 (43,75)	223 (43,90)	0.98
		G		139 (56.50)	528 (53.33)		85 (56,67)	243 (50,41)		54 (56,25)	285 (56,10)	
9p21	rs2383206	AA	Genotypic	24 (21.43)	107 (21,92)	0.58	17 (25,37)	45 (18,99)	0.50	7 (15,56)	62 (24,70)	0.19
		AG		59 (52.68)	233 (47,75)		33 (49,25)	123 (51,90)		26 (57,78)	110 (43,82)	
		GG		29 (25.89)	148 (30,33)		17 (25,37)	69 (29,11)		12 (26,67)	79 (31,47)	
		A	Allelic	107 (47.77)	447 (45.80)	0.59	67 (50.00)	213 (44,94)	0.30	40 (44,44)	234 (46,61)	0.7
		G		117 (52.23)	529 (54.20)		67 (50.00)	261 (55,06)		50 (55,56)	268 (53,39)	
9p21	rs10757278	AA	Genotypic	28 (22.76)	111 (22,79)	0.95	19 (25,33)	60 (25,10)	0.97	9 (18,75)	51 (20,56)	0.71
		AG		64 (50.04)	247 (50,72)		36 (48,00)	118 (49,37)		28 (58,33)	129 (52,02)	
		GG		31 (25.20)	129 (26,49)		20 (26,67)	61 (25,52)		11 (22,92)	68 (27,42)	
		A	Allelic	120 (48.78)	469 (48.15)	0.86	74 (49,33)	238 (238)	0.92	46 (47,92)	231 (46,57)	0.81
		G		126 (51.22)	505 (51.85)		76 (50,67)	240 (240)		50 (52,08)	265 (53,43)	

Table 5. Distribution of allelic and genotypic frequencies for studied polymorphic variants in the group of patients with CAD (with myocardial infarction) and population controls with Bulgarian origin

Locus	Polymorphism	Genotype/ allele	Model	Total			Male			Female		
				MI	Population controls	P value	MI	Population controls	P value	MI	Population controls	P value
9p21	rs7865618	GG	Genotypic	24 (12.06)	84 (16.97)	0.06	13 (9,92)	32 ()13,33	0.32	11 (16,18)	52 (20,39)	0.16
		GA		91 (45.73)	245 (49.49)		66 (50,38)	130 (54,17)		25 (36,76)	115 (45,10)	
		AA		84 (42.21)	166 (33.54)		52 (39,69)	78 (32,50)		32 (47,06)	88 (34,51)	
		G	Allelic	139 (34.92)	413 (41.72)	0.019	92 (35,11)	194 (40,42)	0.16	47 (34,56)	219 (42,94)	0.08
		A		259 (65.08)	577 (58.28)		170 (64,89)	286 (59,58)		89 (65,44)	291 (57,06)	
9p21	rs1537378	TT	Genotypic	27 (16,67)	79 (15,99)	0.64	16 (15,69)	40 (16,60)	0.78	11 (18,33)	39 (15,42)	0.50
		TC		71 (43,83)	237 (47,98)		49 (48,04)	123 (51,04)		22 (36,67)	114 (45,06)	
		CC		64 (39,51)	178 (36,03)		37 (36,27)	78 (32,37)		27 (45,00)	100 (39,53)	
		T	Allelic	125 (38,58)	395 (39,98)	0.66	81 (39,71)	203 (42,12)	0.56	44 (36,67)	192 (37,94)	0.80
		C		199 (61,42)	593 (60,02)		123 (60,29)	279 (57,88)		76 (63,33)	314 (62,06)	
9p21	rs7857345	TT	Genotypic	12 (6,09)	39 (7,89)	0.37	7 (5,43)	13 (5,42)	1	5 (7,35)	26 (10,24)	0.08
		TC		69 (35,03)	192 (38,87)		52 (40,31)	97 (40,42)		17 (25,00)	95 (37,40)	
		CC		116 (58,88)	263 (53,24)		70 (54,26)	130 (54,17)		46 (67,65)	133 (52,36)	
		T	Allelic	93 (23,60)	270 (27,33)	0.16	66 (25,58)	123 (25,63)	0.99	27 (19,85)	147 (28,94)	0.03
		C		301 (76,40)	718 (72,67)		192 (74,42)	357 (74,38)		109 (80,15)	361 (71,06)	
9p21	rs10757274	AA	Genotypic	32 (16,08)	103 (20,81)	0.17	21 (16,03)	57 (23,65)	0.19	11 (16,18)	46 (18,11)	0.23
		AG		100 (50,25)	256 (51,72)		71 (54,20)	125 (51,87)		29 (42,65)	131 (51,57)	
		GG		67 (33,67)	136 (27,47)		39 (29,77)	59 (24,48)		28 (41,18)	77 (30,31)	
		A	Allelic	164 (41,21)	462 (46,67)	0.06	113 (43,13)	239 (49,59)	0.09	51 (37,50)	223 (43,90)	0.18
		G		234 (58,79)	528 (53,33)		149 (56,87)	243 (50,41)		85 (62,50)	285 (56,10)	
9p21	rs2383206	AA	Genotypic	33 (18,33)	107 (21,93)	0.58	22 (18,80)	45 (18,99)	0.99	11 (17,46)	62 (24,70)	0.40
		AG		88 (48,89)	233 (47,75)		60 (51,28)	123 (51,90)		28 (44,44)	110 (43,82)	
		GG		59 (32,78)	148 (30,33)		35 (29,91)	69 (29,11)		24 (38,10)	79 (31,47)	
		A	Allelic	154 (42,78)	447 (45,80)	0.325	104 (44,44)	213 (44,94)	0.90	50 (39,68)	234 (46,61)	0.16
		G		206 (57,22)	529 (54,20)		130 (55,56)	261 (55,06)		76 (60,32)	268 (53,39)	
9p21	rs10757278	AA	Genotypic	40 (20,30)	111 (22,79)	0.655	29 (22,31)	60 (25,10)	0.83	11 (16,42)	51 (20,56)	0.38
		AG		99 (50,25)	247 (50,72)		67 (51,54)	118 (49,37)		32 (47,76)	129 (52,02)	
		GG		58 (29,44)	129 (26,49)		34 (26,15)	61 (25,52)		24 (35,82)	68 (27,42)	
		A	Allelic	179 (45,43)	469 (48,15)	0.36	125 (48,08)	238 (49,79)	0.66	54 (40,30)	231 (46,57)	0.20
		G		215 (54,57)	505 (51,85)		135 (51,92)	240 (50,21)		80 (59,70)	265 (53,43)	

group of patients with MI. Considering the whole group of patients, this impact decreased from 1.4 to 1.29 times, despite the greater number of analyzed cases, and completely disappeared in the group of patients with stable CAD. We hypothesized that this polymorphism was likely associated only with an increased risk of MI, which remained to be confirmed in further studies.

We observed an association between the allele C of rs7857345 and a 1.64-fold increased risk for MI in females (OR 1.64, CI95: 1.03-2.61, p = 0.03). In the males group, no similar trend was observed. For the other studied polymorphisms, no statistically significant association with disease risk was found.

The analysis of the association of the most common haplotypes in the Bulgarian population with the risk of CAD and myocardial infarction development is summarized in Table 6.

Only ATCGGG haplotype showed a slight association with the risk of MI (p = 0.04), but this association was not found after Bonferoni correction (padj = 0.24).

Currently, although polymorphic variants in 9p21 locus do not correlate with levels of conventional risk factors such as arterial blood pressure and lipid levels, our study found a positive association between rs2383206 and triglyceride and LDL cholesterol level. The carriage of polymorphic allele A for this polymorphism was associated with a decrease in triglyceride levels with 0.21 units (ß = -0.21, p = 0.003) and in LDL cholesterol levels with 0.34 units in the general group (ß = -0.34, p = 0.002). Furthermore, carrying the polymorphic T allele for rs10757274 resulted in a decrease of 0.40 units in the LDL cholesterol levels in the male group (ß = -0.40, p = 0.02). There was a gender relationship that was not been reported in the scientific literature up to date (Table 7).

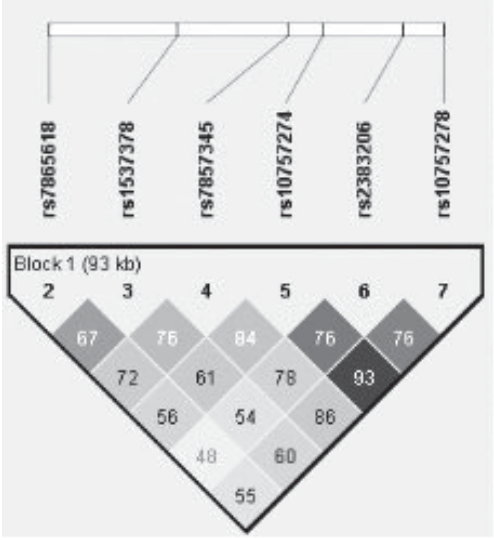

Fig. 1. Diagram of LD structure in the 9p21 region. The solid spine of LD approach in HaploView 4.0 was used to construct the LD block structure

Table 6. Association analysis of the most common haplotypes in the Bulgarian population with the risk of CHD and myocardial infarction

Haplotype						CAD (with and without MI)		CAD (whithout MI)		MI		MI vs CAD (without MI)	
rs7865618	rs1537378	rs7857345	rs10757274	rs2383206	rs10757278	Frequency patients / controls	P	Frequency patients / controls	P value	Frequency patients / controls	P value	Frequency patients / controls	P value
A	C	C	G	G	G	0.37/ 0.33	0.09	0.36/ 0.33	0.31	0.38/ 0.37	0.83	0.38/ 0.33	0.08
G	T	T	A	A	A	0.18/ 0.20	0.27	0.19/ 0.20	0.81	0.17/ 0.19	0.50	0.17/ 0.20	0.24
A	C	C	A	A	A	0.07/ 0.07	0.65	0.08/ 0.07	0.54	0.07/ 0.08	0.75	0.07/ 0.07	0.80
G	T	C	A	A	A	0.06/ 0.07	0.43	0.05/ 0.07	0.40	0.07/ 0.06	0.63	0.06/ 0.07	0.64
A	C	C	G	A	G	0.04/ 0.05	0.20	0.05/ 0.04	0.25	0.05/ 0.05	0.73	0.05/ 0.05	0.53
G	C	C	G	G	G	0.05/ 0.04	0.21	0.02/ 0.03	0.25	0.05/ 0.03	0.36	0.05/ 0.04	0.42
A	T	C	G	G	G	0.03/ 0.03	0.87	0.02/ 0.03	0.32	0.04/ 0.01	0.04	0.04/ 0.03	0.29
A	C	C	G	G	A	0.03/ 0.02	0.40	0.02/ 0.02	0.66	0.03/ 0.03	0.99	0.02/ 0.02	0.61
G	T	T	A	G	A	0.02/ 0.02	0.52	0.02/ 0.02	0.98	0.03/ 0.02	0.51	0.01/ 0.03	0.18
A	C	C	A	G	A	0.01/ 0.03	0.11	0.01/ 0.02	0.61	0.02/ 0.02	0.89	0.03/ 0.02	0.49
A	T	T	A	A	A	0.02/ 0.02	0.97	0.01/ 0.01	0.92	0.02/ 0.01	0.84	0.02/ 0.02	0.95

Table 7. Statistically significant associations of the studied polymorphic variants (at significance level p < 0.05) with the TG and LDL cholesterol levels

Chromosome	Gene	Variantт	Position	B regression coef.	SE	P	Bonferoni correction
Triglicerides							
Total							
9	*9p21*	rs2383206	22115027	-0.21	0.07	0.003	0.018
Male							
9	*9p21*	rs2383206	22115027	-0.23	0.11	0.04	0.24
Female							
9	*9p21*	rs2383206	22115027	-0.18	0.08	0.02	0.12
LDL							
Total							
9	*9p21*	rs2383206	22115027	-0.34	0.11	0.002	0.012
Male							
9	*9p21*	rs2383206	22115027	-0.43	0.16	0.01	0.06
9	*9p21*	rs10757274	22096056	-0.40	0.16	0.02	0.12

Three polymorphic variants – rs10757274, rs2383206 and rs10757278 (Table 8) showed statistically significant association with MI after linear regression analysis and correction for major risk factors for stable CAD and MI, such as BMI, total cholesterol levels, HDL cholesterol, LDL cholesterol, triglycerides, sex and age.

DISCUSSION

The original discovery of the 9p21 locus was done in a group of patients with atherosclerosis, predominantly of European and Asian descent [1, 2]. Replication studies with this locus were conducted for different ethnic groups: Poles [21, 50], Koreans [51, 52], Japanese [51, 53], Chinese [54, 55], Italians [56, 57], Arabs [58, 59], Norwegians [60], Spaniards [61], Turks [62, 63], Pakistanis [64], Indians [65], Iranians [66] and others. Currently, this genome region could be considered as the strongest genetic marker for CAD and MI.

In this association study we found a correlation between the carriage of polymorphic allele for rs7865618 (allele A) at locus 9p21 and the increased risk of CAD (OR 1.29, p = 0.01) and for MI (OR 1.33 p = 0.019). This discovery was proportional to the number of polymorphic alleles. In females, an association between the allele C of rs7857345 and MI was observed (OR 1.64, CI95: 1.03-2.61, p = 0.03).

Schunkert et al. in 2008 investigated the association of locus 9p21 with CAD in 7 case-control stud-

Table 8. Linear regression model in patients with myocardial infarction

Linear regression model B		Unstandardized Coefficients		Standardized Coefficients	t	Sig. Lower Bound	95,0% Confidence Interval for B	
		Std. Error	Beta				Upper Bound	
	(Constant)	0,439	0,153		2,861	0,005	0,136	0,741
	Age	0,013	0,001	0,438	9,055	0,000	0,010	0,016
	SEX	-0,135	0,040	-0,134	-3,359	0,001	-0,214	-0,056
	Chol	0,260	0,165	0,516	1,575	0,117	-0,065	0,585
	HDL	-0,235	0,163	-0,174	-1,446	0,150	-0,556	0,085
	LDL	-0,242	0,158	-0,478	-1,534	0,127	-0,554	0,069
	TG	0,095	0,052	0,156	1,843	0,067	-0,007	0,197
	rs7865618	0,039	0,033	0,053	1,186	0,237	-0,026	0,103
	rs10757274	-0,144	0,033	-0,195	-4,392	0,000	-0,209	-0,080
	rs7857345	0,018	0,035	0,025	0,517	0,606	-0,051	0,087
	rs1537378	0,037	0,033	0,053	1,121	0,263	-0,028	0,102
	rs2383206	0,100	0,025	0,170	4,033	0,000	0,051	0,148
	rs10757278	0,143	0,027	0,242	5,257	0,000	0,090	0,197

Dependent Variable: Diagnosis

ies and undertook a meta-analysis. A single-nucleotide polymorphism (SNP), rs1333049, representing the 9p21.3 locus, (the rs1333049 SNP was also in strong linkage disequilibrium with the rs10757278 SNP [67] with D' statistic, 0·794; r statistic, 0·726 and P < 0·01.) was genotyped in 7 case-control studies involving a total of 4645 patients with myocardial infarction or CAD and 5177 controls. The mode of inheritance was determined. In addition, in 5 of the 7 studies, they genotyped 3 additional SNPs to assess a risk-associated haplotype (ACAC). Finally, a meta-analysis of the present data and previously published samples was conducted. A limited fine mapping of the locus was performed. The risk allele (C) of the lead SNP, rs1333049, was uniformly associated with CAD in each study ($p < 0.05$). In a pooled analysis, the odds ratio per copy of the risk allele was 1.29 (95% confidence interval, 1.22 to 1.37; $p = 0.0001$). Haplotype analysis further suggested that this effect was not homogeneous across the haplotypic background (test for interaction, P = 0.0079). An autosomal-additive mode of inheritance best explained the underlying association. The meta-analysis of the rs1333049 SNP in 12,004 cases and 28,949 controls increased the overall level of evidence for association with CAD to P = 6.04x10(-10) (odds ratio, 1.24; 95% confidence interval, 1.20 to 1.29). Genotyping of 31 additional SNPs in the region identified several with a highly significant association with CAD, but none had predictive information beyond that of the rs1333049 SNP [68].

In their study Koch et al. (2011) examining genetic risk of MI at Ch9p21,t and they found an association with rs7865618, rs1537378, rs10811650, rs1333040, rs7857345, rs10757274, rs2383206, rs1333045, rs10757278, and rs1333049. Relations of the same SNPs to MI, CAD, or CHD were observed in prior studies [1, 2, 51, 53, 68-73]. This is in accordance with the results obtained in our study.

Yayla et al. in 2016 aimed to evaluate the impact of rs10757274 and rs2383206 polymorphisms in chromosome 9p21 on the presence and severity of CAD in a Turkish population. A total of 646 patients who underwent coronary angiography were included in this study. Coronary vessel score and Gensini score were calculated to assess the angiographic severity of CAD. Alleles of AA, AG, and GG were determined for rs10757274 (polymorphism-1) and rs2383206 (polymorphism-2) polymorphisms located in chromosome 9p21 from the blood samples. There was a significant difference between the alleles in polymorphism-1 in the presence of coronary artery disease (38.9% in AA, 48.0% in GG and 56.4% in AG, $p = 0.017$) [62].

The genetic variants associated with an increased atherosclerotic risk such as CAD and MI are localized in the genome region that produces long, non-coding antisense RNA called ANRIL (antisense RNA in the INK4 locus). In individuals with reduced risk of cardiovascular damage, shearing of this RNA occurred in two smaller fragments: one short linear and one circular RNA (cANRIL). In humans at an increased risk for atherosclerotic disease, only the long ANRIL RNA was found to inhibit gene expression of the INK4/ARF locus more effectively. The expression-associated INK4/ARF region prevents the formation of atherosclerotic plaques and therefore people in whom this locus was suppressed were more susceptible to atherosclerotic disease [74].

Identification of 9p21 region as a genetic marker for CAD is of great significance in understanding the genetic basis of cardiovascular disease. At present, there is no other part of the human genome showing such a strong association with the cardiac ischemia manifestation, as well as with many other consequences of cardiovascular damage such as atherosclerotic plaques in the carotid artery [29], stroke [30-33], peripheral arterial disease [34-36], heart failure [37] and cardiovascular mortality [38, 39], also intracranial and abdominal aortic aneurysms [40-42], coronary stenosis [43, 44], and aortic calcification [45]. All this suggests a more general role of 9p21 locus in vascular pathology. Genetic changes in the 9p21 locus are a risk factor for CAD in all ethnic groups except African-Americans [28].

The potential association between polymorphic variants in the 9p21 locus and atherosclerosis may be explained by the antiproliferative action of cyclin-dependent kinase inhibitors that is known to be suppressed in individuals with the 9p21 risk allele [28]. Cell proliferation and apoptosis play an important role in atherogenesis and there is evidence that genes in the INK4/ARF may be associated with atherogenesis. Folkersen et al. reported an increased CDKN2A, CDKN2B and MTAP gene expression in normal and atherosclerotic coronary arteries [75]. However, the expression levels of these genes in vascular tissue did not show a clear correlation with 9p21 locus, which negates the role of altered expression of these genes in determining atherosclerosis sensitivity until further data is aquired. Many other studies have examined the relationship between CDKN2A, CDKN2B, and MTAP expression in peripheral blood cells, but most of the results are negative [75-78].

Some functional studies have been performed focusing on the differential expression of antisense non-coding RNA from INK4 (ANRIL), which is transcribed by 9p21, as well as from the neighboring protein-cod-

ing genes. The leading concept is that ANRIL might be a regulator of epigenetic modification and thus modulate cardiovascular risk [79, 80].

Genetic variations can affect the phenotype either by altering the nature of the gene product (quality) or its expression (quantity). Most risk variants in 9p21 are located in non-coding regions away from protein-coding genes. This suggests that their effect is likely due to their impact on the gene expression of one or more genes located more closely in the genome.

Some studies indicate that the 9p21 locus is involved in the initiation of atherosclerosis and is not associated with the progression or severity of atherosclerosis [5], while other studies have the opposite assumptions [12, 17]. However, all studies are unanimous in concluding that polymorphic variants in 9p21 indicate a higher risk of coronary disease at early age (55 years in male and 60 years in female) [5].

Another possible explanation for the association between the 9p21 and an increased risk of atherosclerosis is the presence of multiple enhancers in the CDKN2B-AS region, which are responsible for the elevated expression of cell proliferation-inducing genes. In a study of Harismendy et al., 33 enhancers were identified within the sequence [81].

All of this explains the obtained association between the carrier of polymorphic alleles for 9p21 locus and the risk of CAD and MI in this study. However, an enlargement of the studied groups is needed to confirm the identified relations.

Ethical standards: *All procedures performed in the studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee (Ethics Committees of the Medical University of Sofia and the Medical University of Pleven) and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.*

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