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Gain of function variants in *PCSK9* gene in high risk patients after myocardial infarction with increased lipoprotein (a) values and treated with statins

Sabina Ugovšek[1,2]✉, Andreja Rehberger Likozar[3], Tina Levstek[4,5], Katarina Trebušak Podkrajšek[4,5], Janja Zupan[6], Slobodan Antonić[7] & Miran Šebeštjen[2,3,8]

The proprotein convertase subtilisin/kexin type 9 (PCSK9) regulates lipid metabolism, inflammation and haemostasis. Pathogenic gain-of-function variants in the *PCSK9* gene are causative of autosomal-dominant form of familial hypercholesterolemia, while several *PCSK9* alleles have been associated with elevated levels of low-density lipoprotein cholesterol (LDL-C) and an increased risk of cardiovascular disease. Elevated lipoprotein(a) (Lp(a)) levels, regardless of LDL-C levels, as well as functional and morphological changes in the arterial vessel wall predict future cardiovascular events. In our study, we aimed to identify whether treatment with statins nullifies the effect of four gain-of-function gene variants in *PCSK9* on lipoproteins and arterial wall properties in high-risk post-myocardial infarction patients with severely elevated Lp(a) levels. We included 68 patients after myocardial infarction with elevated Lp(a) levels on maximal statin therapy. Biochemical analysis of lipid parameters and total PCSK9 levels were performed. Arterial wall properties were measured by ultrasound. Genotyping was performed for four gain-of-function, i.e. rs11206510, rs2479409, rs2479408 and rs1711503, and one intergenic, rs11591147 *PCSK9* single nucleotide polymorphisms. The results showed no association between studied *PCSK9* gene variants and lipid parameters, PCSK9 levels and arterial wall properties in our patient cohort. Clinical, biochemical and arterial wall parameters did not differ between the group with lower compared to group with higher number of *PCSK9* alleles. Our results suggest that in high-risk patients after myocardial infarction with increased Lp(a) levels treated with statins the studied *PCSK9* gain-of-function gene variants are associated neither with lipoproteins nor with arterial wall properties.

Keywords *PCSK9* polymorphisms, Lipoprotein(a), Endothelial dysfunction, Total PCSK9 levels

Proprotein convertase subtilisin/kexin type 9 (PCSK9) is an enzyme that is synthesized to the greatest extent in hepatocytes, but also in the kidneys, small intestine, endothelial cells and macrophages[1]. The primary function of PCSK9 is binding to low-density lipoprotein receptors (LDLR) and thereby shortening their half-life, which in turn leads to an increased concentration of low-density lipoprotein cholesterol (LDL-C) and an increased risk of cardiovascular events[2]. Besides the influence on the concentration of LDL-C, PCSK9 also affects inflammatory risk factors and factors of haemostasis. All of these risk factors, similar to LDL-C are involved in all phases of the

[1]Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia. [2]Department of Cardiology, University Medical Centre Ljubljana, Zaloška cesta 7, 1000 Ljubljana, Slovenia. [3]Department of Vascular Diseases, University Medical Centre Ljubljana, Zaloška cesta 7, 1000 Ljubljana, Slovenia. [4]Laboratory for Translational Medical Biochemistry, Institute of Biochemistry and Molecular Genetics, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia. [5]Clinical Institute for Special Laboratory Diagnostics, University Children's Hospital, University Medical Centre Ljubljana, Vrazov trg 1, 1000 Ljubljana, Slovenia. [6]Faculty of Pharmacy, University of Ljubljana, Aškerčeva cesta 7, 1000 Ljubljana, Slovenia. [7]Department of Neurology, University Medical Centre Ljubljana, Zaloška cesta 7, 1000 Ljubljana, Slovenia. [8]Department of Internal Medicine, Faculty of Medicine, University of Ljubljana, Vrazov trg 2, 1000 Ljubljana, Slovenia. ✉email: ugovsek.sabina@gmail.com

atherosclerotic process, from endothelial dysfunction to atherosclerotic plaque rupture and the resulting acute event[3].

The blood levels of PCSK9 vary greatly between individuals, largely due to genetic factors in the *PCSK9* gene, which is located on chromosome 1p32[4]. Pathogenic gain-of-function *PCSK9* variants were reported to result in an autosomal-dominant form of familial hypercholesterolemia[5,6]. Additionally, several variants were reported to be associated with increased LDL-C concentrations and also a higher incidence of cardiovascular diseases, while loss-of-function variants are associated with life-long reduced LDL-C concentrations and a reduced risk of cardiovascular events[7]. The most studied variant in the *PCSK9* gene is single nucleotide polymorphism (SNP) rs11591147, where an arginine to leucine substitution occurs at the amino acid position 46 (R46L) and is present in approximately 3.2% of the white population[8]. Previous studies have shown that carriers of the leucine allele of the rs11591147 have a greatly reduced concentration of PCSK9 and consequently LDL-C concentration, and a lower risk of cardiovascular events[7,9–11]. Another variant rs11206510 in the *PCSK9* gene was shown to be associated with increased LDL-C levels regardless of prior statin treatment. At the same time, carriers of the variant had a lower chance of achieving target LDL-C levels and a higher risk of a future cardiovascular event compared to non-carriers, given the same statin treatment[12]. A recent study in the Mexican population showed that among apparently healthy individuals, carriers of rs2479409 in the *PCSK9* gene had more often subclinical atherosclerotic conditions measured as coronary artery calcium (CAC) score compared to non-carriers[13]. At the same time, the two groups differed only in the concentration of triglycerides, but not in LDL-C. However, carriers of rs2479409 had more pronounced risk factors in the context of metabolic syndrome, namely, greater insulin resistance and the concentration of interleukin (IL) 1ß[13]. Polymorphisms rs2479408 and rs1711503 were associated with ischemic stroke in certain populations in China[14]. Nonetheless, both SNPs also differed from control groups in some other risk factors, for instance LDL-C concentration and the frequency of diabetes, which are directly related to the concentration PCSK9. Thus, we cannot claim with absolute certainty that the mentioned polymorphisms are directly related to the incidence of ischemic stroke. It is more likely that it is an indirect effect of the increased concentration of PCSK9 on the previously mentioned risk factors[14]. Another polymorphism in *PCSK9*, rs2479415, is a common SNP that has been associated with increased hepatic *PCSK9* mRNA levels and is an independent determinant of increased levels of both, PCSK9 and LDL-C in a healthy middle-aged white population[2]. However, to the best of our knowledge no previous studies evaluated the association between rs2479415 and the incidence of cardiovascular events, or at least atherosclerotic changes.

Additionally, severely elevated levels of lipoprotein(a) (Lp(a))[15], and endothelial dysfunction, a first detectable functional change in the atherosclerotic process[16], are an independent predictor of future cardiovascular events in patients with coronary artery disease (CAD) and in patients at risk for CAD[17]. Increased carotid intima-media thickness (c-IMT) are morphological changes following functional changes measured as increased pulse wave velocity (PWV) and decreased flow-mediated dilation (FMD) of the arterial vessel wall which were all found to be predictors of future cardiovascular events[18].

All the aforementioned polymorphisms in *PCSK9* gene are associated with the PCSK9 and LDL-C levels and thus at least indirectly to the properties of the vessel wall. In the current study, we aimed to identify whether treatment with statins nullifies the effect of four gain-of-function gene variants in *PCSK9* on lipoproteins and arterial wall properties in high-risk post-myocardial infarction patients with severely elevated Lp(a) levels.

Statistical analysis

The Hardy–Weinberg equilibrium for all SNPs was assessed using chi-squared tests. Haplotype frequencies were calculated using the PHASE software (https://stephenslab.uchicago.edu/phase/download.html). Kolmogorov–Smirnov tests were used to define variables showing normal distributions, with these data expressed as means and standard deviations. The non-normally distributed variables were expressed as medians and range (lower and upper quartiles). A general linear model using age and body mass index as a covariate was used to determine the relationship between genotype and haplotype groups and Lp(a) levels. The differences between genotype subgroups were calculated with one-way ANOVA or Kruskal Wallis test. The differences between subgroups with different numbers of risk alleles were calculated with Student's t test or Mann-Whitney test. Statistical analysis was performed using IBM SPSS Statistics for Windows, (Version 25.0. Armonk, NY, USA: IBM Corp.) and GraphPad Prism version 6 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com, accessed on 28 August 2024). p values < 0.05 were considered as statistically significant.

Patients and methods

Patients

We included a sub-cohort of the patients included in our previous study[19]. Briefly, 68 patients at least 6 months after myocardial infarction and with severely elevated Lp(a) levels were included. All patients were treated with statins at the highest tolerated doses, along with ezetimibe where needed as well as beta blockers, angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers and antiplatelet drugs for at least 8 weeks before the study. A study design is shown in Fig. 1. All the procedures performed in this study that involved human participants were carried out in accordance with the ethical guidelines of the 1964 Declaration of Helsinki. Approval for this study was obtained from the National Medical Ethics Committee of the Republic of Slovenia (KME 0120–357/2018/8; date of approval: 23 April 2019 and KME 0120–317/2021/3; date of approval: 2 August 2021). All patients signed a written informed consent form prior to inclusion in the study.

Biochemical analysis

Blood for laboratory analysis was drawn in the morning after 12 h of fasting. We collected samples from the antecubital vein into 5 mL vacuum-sealed tubes containing clot activator (Vacutube; LT Burnik, Skaručna, Slovenia). We centrifuged the blood at 2000 g for 15 min to separate the serum. In the fresh serum samples,

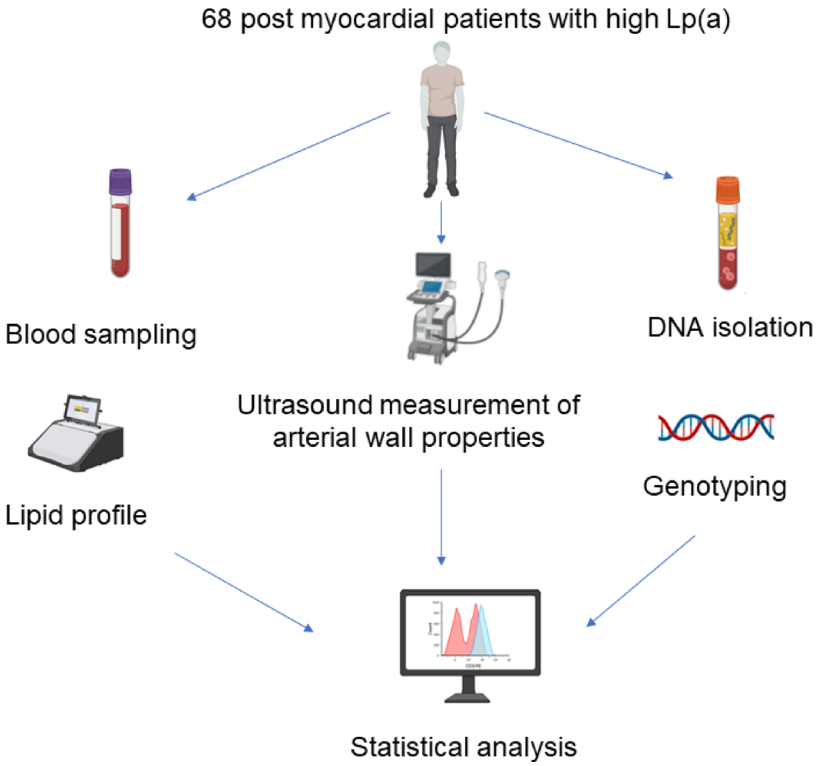

Fig. 1. Design of the study.

we measured total cholesterol (TC), triglycerides (TG), high-density lipoprotein cholesterol (HDL-C), apolipoprotein A1 (apoA1), and apolipoprotein B100 (apoB) by standard colorimetric or immunologic assays. We used an automated biochemical analyzer (Fusion 5.1; Ortho-Clinical Diagnostics, Raritan, NJ, USA). The same biochemical analyser was used to determine Lp(a) with the Denka reagent (Randox, Crumlin, UK). Because of the apo(a)-isoform-insensitive antibodies used in the reagent, the bias associated with apo(a) size was minimal. Concentration of total PCSK9 was measured using a classic sandwich ELISA (R&D Systems, Inc., Minneapolis, MN, USA). The Friedewald formula[20] was used to calculate LDL-C.

Brachial artery flow-mediated dilation

Endothelial function was assessed by means of flow-mediated dilatation (FMD) on the brachial artery according to the guidelines[21]. Measurements for each subject were performed at the same time of the day, after a 10-minute rest in a quiet and temperature-controlled room. Subjects rested in supine position with the right arm extended and immobilized with foam, supported at an angle of approximately 80° from the torso. Blood pressure was recorded with an automated sphygmomanometer (Welch Allyn Speidel & Keller OSZ Digital Blood Pressure System) on the contralateral arm. Another blood pressure cuff was placed around the right forearm. Brachial artery diameter was visualized 5–10 cm above the antecubital fossa. The probe was locked in a stereotaxic instrument. The echo-machine continuously tracked and recorded the brachial artery diameter. After measurements of baseline brachial artery diameter (1 min), the forearm blood pressure cuff was inflated at least 50 mmHg above the patients' systolic blood pressure for 4 min, producing arterial occlusion. After the occlusion period, the cuff was rapidly deflated, inducing reactive hyperemia, and the brachial artery diameter was recorded for 3 min. At the end of the measurement, the machine automatically provided the values of baseline and maximal brachial diameter and FMD (the percentage of change from the baseline diameter of the brachial artery during reactive hyperemia). All images were recorded and saved onto the external hard drive.

Arterial stiffness parameters assessment

All measurements were performed on the right common carotid artery with a linear vascular probe (working frequency of 5–13 MHz) as described in Rehberger et al.[19] Testing was performed with subjects comfortably lying in supine position with head elevation of around 45° and side tilt of 30° to the left in a quiet room with the air temperature of 22–24 °C. The Aloka prosound α7 (Hitachi Aloka Medical, Ltd., Japan) ultrasound machine was also equipped with special software with an integrated high-resolution eTracking system (Hitachi Aloka, Wallingford, CT, USA) for automatic determination of the stiffness parameters of common carotid artery through the analysis of pulse wave. The echo-tracker's cursor-pair was placed onto the anterior and posterior walls of the common carotid artery, approximately 1 to 2 cm proximal to the carotid bulb. Pressure waveforms were noninvasively obtained using arterial diameter change waveforms automatically calibrated based on systolic and diastolic blood pressure values measured as described above. The carotid artery local stiffness (β-stiffness)

and values of local PWV were automatically calculated as a mean of six beats. The measurement was repeated six times and the mean value was taken.

Intima-media thickness

All measurements were performed on both sides of common carotid artery and internal carotid artery according to the guidelines[22]. Plaques in the bulbs were given descriptively; whether plaque was present in the bulbs or not. The presence of plaque means c-IMT of more than 1.1 cm. Testing was performed with subjects in supine position and under the same conditions as described above on the Vivid E95 Ultrasound machine. Measurements were performed 2 cm proximal to the bulbs of the common carotid artery and 1.5 cm distally from the ostium of the internal carotid artery. The thickness of the intima-media was automatically calculated using the EchoPac 230 program as the mean value and standard deviation in the marked part of the intima-media of the carotid artery.

Genotyping of single nucleotide polymorphisms in PCSK9 gene

Genomic DNA was isolated from buffy coat using the FlexiGene DNA kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. Five SNPs in the *PCSK9* gene were selected, namely rs11206510, rs2479409, rs2479408, rs1711503 and rs11591147. Genotyping was performed using predesigned TaqMan SNP Genotyping Assays (Thermo Fisher Scientific, Waltham, MA, USA) and TaqMan® Universal PCR Master Mix (Applied Biosystems, Waltham, MA, USA) according to the manufacturer's instructions in a QuantStudio 7 Flex Real-Time PCR System (Applied Biosystems, Waltham, MA, USA). Genotyping was performed blind to all clinical data and was repeated randomly on 10% of the samples to check the reliability.

Results

Patients' characteristic

The clinical and laboratory characteristics of the patients are presented in Table 1. The majority of the patients were males (88%), 4 (5.8%) were active smokers and 8 (11.7%) were patients with type 2 diabetes. All risk factors were controlled, except for LDL-C and Lp(a) values, despite the maximum tolerated statin dose and, if necessary, ezetimibe[23].

Genotype frequencies

Samples from all patients were genotyped for 5 SNPs in the *PCSK9* gene, namely 4 gain-of function SNPs (rs11206510, rs2479409, rs2474908 and rs1711503) that were previously associated with increased concentration of PCSK9 and LDL-C[12–14] and intergenic rs11591147. The frequency of the genotypes is shown in Fig. 2. Genotype frequencies for all SNPs were in Hardy–Weinberg equilibrium ($p > 0.05$). The distribution frequencies for rs11206510 and rs2479409 in our patient cohort are comparable to the European population, while the frequencies for G genotype are higher in our cohort than in European population, but we do not know whether the difference is statistically significant[24].

Associations between PCSK9 SNPs, biochemical and arterial wall properties parameters

We did not find any differences in the values of TC, HDL-C, LDL-C, TG, apoA1, apoB, Lp(a) and total PCSK9 between the genotype groups (Table 2). Likewise, no difference was found between the subgroups of same genotype in the parameters of the vascular wall properties (Table 3).

Given that higher number of risk alleles is associated with increased concentration of LDL-C[7], we further aimed to verify if there are differences between the two groups of patients, namely patients with low number (5 to 7) versus patients with high (7 to 9) risk alleles. The number of risk alleles was calculated as sum of

Parameter	Value ($n = 68$)
Males/females (%)	60/8 (88/12)
Age (years)	51.7 ± 8.7
Body mass index (kg/m^2)	28.7 ± 8.7
Systolic blood pressure (mmHg)	128 ± 15
Diastolic blood pressure (mmHg) Blood glucose (mmol/l)	77 ± 9 5.9 ± 1.4
Total cholesterol (mmol/L)	4.24 ± 0.83
HDL cholesterol (mmol/L)	1.20 ± 0.28
LDL cholesterol (mmol/L)	2.35 ± 0.70
Triglycerides (mmol/L)	1.65 ± 0.79
Lipoprotein(a) (mg/L)	1431 (11207–1783)
Apolipoprotein B (g/L)	0.81 ± 0.22
Apolipoprotein A1 (g/L)	1.34 ± 0.19
Total PCSK9 (ng/mL)	285.7 (210.0–401.3)

Table 1. Clinical and biochemical variables of the patients at baseline of the study. HDL, high-density lipoprotein; LDL, low-density lipoprotein; PCSK9, proprotein convertase subtilisin/kexin type 9. Values are presented as means ± standard deviations or medians (lower–upper quartile).

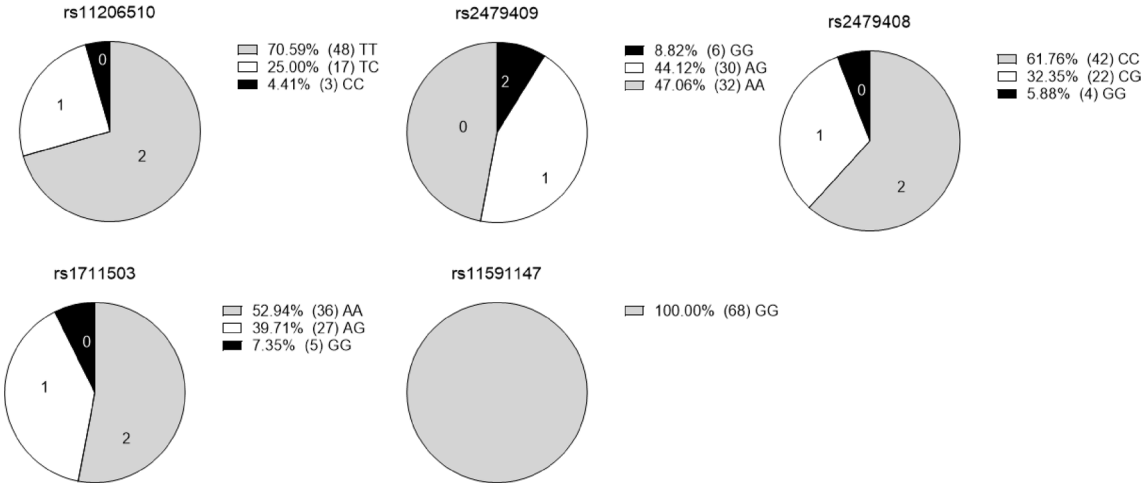

Fig. 2. Genotype frequency distributions Values are presented as counts (percentage). Score is defined as the presence of the risk allele (2, two risk alleles; 1, one risk allele; 0, no risk allele).

scores (defined in Fig. 2) for all SNPs. Our results showed no differences in the concentration of biochemical parameters, nor in the parameters of the vascular wall properties between the groups with low and high number of risk alleles (Table 4). However, the triglyceride levels were higher in patients with high number of risk alleles. However, all our patients had at least 5 risk alleles in a possible range between 0 and 10.

Moreover, we also tested if the properties of the arterial vessel wall were associated with PCSK9 concentrations. The results showed no statistically significance (Fig. 3).

Discussion

In our study, we genotyped 4 different gain-of-function SNPs (rs11206510, rs2479409, rs2474908 and rs1711503) that were previously associated with an increased risk of coronary artery disease[12–14] and one intergenic rs11591147.

The distribution frequencies for rs11206510 and rs2479409 in our patient cohort are comparable to the European population, despite the fact that we included only high-risk patients[24]. For rs2479408 and rs1711503, the frequencies for G genotype are higher in our cohort than in European population, but we cannot claim whether the difference is statistically significant[24].

On the other hand, we did not detect the presence of the T genotype in rs11591147, the frequency of which in the Caucasian population is 3.2%[8]. The result is not surprising, since it is the loss-of-function allele, which is associated with a lower concentration of LDL-C and a lower incidence of CAD[25], and only patients who had already suffered a myocardial infarction were included in our research. In a study by Cohen et al.[7], it was shown that in Caucasians the presence of the T allele is associated with a 15% lower concentration of LDL-C and a 47% lower risk of ischemic heart disease. At the same time, there were no differences in the prevalence of other risk factors compared to non-carriers, from which it could be concluded that PCSK9 has other proatherogenic effects in addition to its effect on LDL-C concentration. In the same study, this difference was even more evident in Afro-Americans, where with a 28% reduction in LDL-C, the risk of developing ischemic heart disease was as much as 88% lower. We have to keep in mind that carriers and non-carries of the T genotype in rs11591147 also differed significantly in the prevalence of arterial hypertension. Factors that could easily contribute to a reduced risk of ischemic heart disease in PCSK9 R46L carriers, in addition to a lower concentration of LDL-C, are a lower concentration of Lp(a), secretory phospholipase A2 and lipoprotein-associated phospholipase A2 activity[26].

There is little data on the association of the properties of the arterial vessel wall with individual *PCSK9* polymorphisms. Almost all data refer to c-IMT, but there are only few data on FDM or PWV. Most of the data are available for rs11591147, in which carriers of the T allele have significantly lower concentrations of PCSK9[27], and LDL-C and, more importantly, a lower risk of future cardiovascular events[28]. The SPREDIA study, performed on 1188 individuals without clinically evident cardiovascular disease, found that carriers of the T allele had statistically significant lower c-IMT than non-carriers, despite no differences in LDL-C concentration between the groups[10]. This difference remained significant even after the results were balanced for sex, age, presence of arterial hypertension and diabetes mellitus, however the statin treatment was not considered a covariable. In both groups (carriers and non-carriers), the same proportion of patients were treated with statins. This fact is extremely important because statin treatment greatly changes the interrelationships between the concentrations of PCSK9, LDL-C and the properties of the arterial vessel wall. Treatment with statins, regardless of the dose or type of statin, significantly increases the PCSK9 concentration and, on the other hand, decreases the LDL-C concentration, which significantly changes arterial vessel wall properties[29–32]. Interestingly, we found no carriers of T allele in our research where only patients after a myocardial infarction were included, which confirms that these are very high-risk patients. One of the reasons for increased risk for CAD in our patient cohort could also be high concentration of Lp(a) that was as an inclusion criterion in our study. The carriers of the T allele rs11591147 have a significantly lower concentration of Lp(a) compared to those without the T allele[26]. The

Parameter	Genotype subgroups			
rs11206510	**T/T**	**T/C**	**C/C**	***p* value**
TC (mmol/L)	4.21 ± 0.87	4.36 ± 0.75	4.16 ± 0.33	0.938
LDL-C (mmol/L)	2.27 ± 0.71	2.39 ± 0.73	2.52 ± 0.22	0.921
HDL-C (mmol/L)	1.19 ± 0.30	1.24 ± 0.26	1.12 ± 0.26	0.728
TG (mmol/L)	1.39 (1.06–2.09)	1.53 (1.09–2.20)	1.10 (0.79–/)	0.458
Lp(a) (mg/L)	1445(1219–1783)	1401 (1179–1905)	1267 (837–/)	0.684
rs2479409	**G/G**	**A/G**	**A/A**	
TC (mmol/L)	4.52 ± 1.05	4.2 ± 0.88	4.18 ± 0.74	0.979
LDL-C (mmol/L)	2.40 ± 0.87	2.30 ± 0.76	2.31 ± 0.62	0.976
HDL-C (mmol/L)	1.10 ± 0.21	1.17 ± 0.28	1.25 ± 0.30	0.312
TG (mmol/L)	2.25 (1.63–3.01)	1.63 (1.16–2.17)	1.25 (0.94–1.73)	0.089
Lp(a) (mg/L)	1522 (1247–1722)	1372 (1219–1572)	1479 (1162–1930)	0.614
PCSK9 (ng/mL)	327 (154–377)	321 (229–393)	275 (195–418)	0.558
rs2479408	**C/C**	**C/G**	**G/G**	
TC (mmol/L)	4.29 ± 0.83	4.07 ± 0.85	4.69 ± 0.45	0.130
LDL-C (mmol/L)	2.37 ± 0.72	2.15 ± 0.66	2.70 ± 0.66	0.553
HDL-C (mmol/L)	1.16 ± 0.23	1.29 ± 0.34	1.15 ± 0.44	0.400
TG (mmol/L)	1.46 (1.07–2.24)	1.35 (1.00–1.70)	2.01 (1.17–2.38)	0.180
Lp(a) (mg/L)	1388 (1205–1778)	1444 (1185–1962)	1560 (1018–1893)	0.575
PCSK9 (ng/mL)	283 (210–352)	300 (204–474)	264 (159–622)	0.357
rs1711503	**A/A**	**A/G**	**G/G**	
TC (mmol/L)	4.23 ± 0.81	4.28 ± 0.90	4.15 ± 0.59	0.910
LDL-C (mmol/L)	2.32 ± 0.69	2.34 ± 0.76	2.09 ± 0.46	0.908
HDL-C (mmol/L)	1.23 ± 0.29	1.19 ± 0.29	1.02 ± 0.14	0.322
TG (mmol/L)	1.30 (1.00–1.81)	1.41 (1.17–2.13)	2.36 (1.44–3.08)	0.311
Lp(a) (mg/L)	1454 (1222–1930)	1378 (1072–1594)	1461 (1125–1590)	0.307
PCSK9 (ng/mL)	274 (216–426)	319 (199–356)	297 (141–392)	0.827

Table 2. Lipid parameters and PCSK9 serum levels in genotype subgroups. TC, total cholesterol; LDL-C, low-density lipoprotein cholesterol; HDL-C, high-density lipoprotein cholesterol; TG, triglycerides; Lp(a), lipoprotein(a); PCSK9, proprotein convertase subtilisin/kexin 9. Values are presented as means ± standard deviations or medians (lower–upper quartile). One-way ANOVA was used to test the differences between the genotype subgroups. *P* values < 0.05 were considered as statistically significant.

research by Zamarron-Lincon et al.[13] showed that LDL-C levels did not differ between the individual rs2479409 genotypes, neither in the control group nor in the group with subclinical atherosclerosis, defined as a CAC score greater than zero. However, rs2479409 was associated with parameters of cardiometabolic syndrome (insulin resistance, hyperinsulinemia, and IL-1ß and IL-10 concentrations), which is a risk factor for progression of atherosclerosis[33]. These risk factors have been shown to be predictors of both, functional and morphological properties of the arterial vessel wall[34]. Thus, rs2479409 might influence other risk factors, which are not solely related to its primary function in the metabolism of LDL-C.

In the LIFE-Heart study, where more than 3000 patients with suspected and confirmed coronary artery disease were included and 38% of them received statins, c-IMT was associated with rs2479409 in the entire study population[11]. This association was much more pronounced in the subgroup that did not receive statins, however there are no data on the association of rs2479409 in the statin-treated group. In any case, this connection was less pronounced, we do not know if it was statistically significant. However, we must be aware that the two groups of patients differed in several risk factors that were found to be associated with PCSK9 concentration[11]. In the same study, the association between the rs2479409 and c-IMT was very clear in the whole group of patients, while this association was significantly less pronounced, although still statistically significant, in the group of patients not treated with statins. It might be that this association was also statistically significant in the group of patients receiving statins, however the authors provide no data. In our research, we did not find any differences in the properties of the arterial vessel wall nor were there any differences between the concentrations of the measured lipid parameters and total PCSK9 between rs2479409, rs1711503 and rs11206510 genotype subgroups.

Parameter	Genotype subgroups			*p* value
rs11206510	**T/T**	**T/C**	**C/C**	
FMD (%)	11.8 ± 5.9	8.5 ± 7.5	10.0 ± 5.4	0.184
Beta stiffness	5.8 ± 1.4	5.8 ± 1.3	5.5 ± 1.6	0.934
PWV β (m/s)	5.3 ± 0.8	5.3 ± 0.7	4.9 ± 0.7	0.694
c-IMT (mm)	0.64 ± 0.1	0.67 ± 0.1	0.72 ± 0.3	0.254
rs2479409	**G/G**	**A/G**	**A/A**	
FMD (%)	10.6 ± 5.4	10.8 ± 7.4	11.1 ± 5.7	0.979
Beta stiffness	6.0 ± 1.5	5.5 ± 1.1	6.1 ± 1.6	0.319
PWV β (m/s)	5.5 ± 0.8	5.1 ± 0.7	5.4 ± 0.8	0.228
c-IMT (mm)	0.66 ± 0.07	0.65 ± 0.12	0.65 ± 0.10	0.948
rs2479408	**C/C**	**C/G**	**G/G**	
FMD (%)	10.2 ± 6.1	12.3 ± 7.2	11.3 ± 4.7	0.431
Beta stiffness	5.9 ± 1.6	5.6 ± 1.0	6.3 ± 1.1	0.591
PWV β (m/s)	5.3 ± 0.8	5.2 ± 0.7	5.5 ± 0.6	0.652
c-IMT (mm)	0.65 ± 0.1	0.64 ± 0.1	0.67 ± 0.03	0.928
rs1711503	**A/A**	**A/G**	**G/G**	
FMD (%)	11.6 ± 6.6	10.2 ± 6.3	9.8 ± 5.6	0.637
Beta stiffness	6.1 ± 1.5	5.5 ± 1.1	5.7 ± 1.5	0.296
PWV β (m/s)	5.4 ± 0.8	5.1 ± 0.6	5.2 ± 0.6	0.183
c-IMT (mm)	0.65 ± 0.10	0.65 ± 0.1	0.66 ± 0.1	0.948
rs11591147	**G/G**	**G/T**	**T/T**	
FMD (%)	10.9 ± 6.4	/	/	
Beta stiffness	5.8 ± 1.4	/	/	/
PWV β (m/s)	5.3 ± 0.7	/	/	/
c-IMT (mm)	0.65 ± 0.1	/	/	/

Table 3. Arterial wall properties in genotype subgroups. FMD, flow-mediated dilatation; PWV, pulse wave velocity; c-IMT, intima-media thickness. Values are presented as means ± standard deviations or medians (lower–upper quartile). One-way ANOVA was used to test the differences between the genotype subgroups. *p* values < 0.05 were considered as statistically significant.

To the best of our knowledge there are no previous studies on the influence of these three *PCSK9* polymorphisms on any of the arterial vessel properties wall measured in our study.

In our study, we also compared the number or risk alleles combined from all 5 studied SNPs. The presence of both alleles that were previously associated with risk for CAD was scored with 2, and the absence of both risk alleles with 0, while the heterozygous were scored as 1. All our patients possessed a sum of scores of at least 5, while none of our patients reached maximum score, i.e. 10. This means that each of our patients had at least 5 alleles that contribute to elevated LDL-C levels via increased PCSK9 concentration. Considering that each of the SNPs explains only an extremely small part of the variability in both, PCSK9 and LDL-C levels, the number of alleles might be more informative in the case of PCSK9 and LDL-C levels. Except for triglyceride concentration, we did not find any differences in the levels of lipoproteins, nor in the properties of the arterial vessel wall between the groups of patients with low (5–7) versus high (8–9) number of risk alleles. There could be several explanations for this finding, one of them is treatment with statins. All of our patients received either atorvastatin or rosuvastatin, and regardless of the type and dose of the statin, PCSK9 levels increased significantly while LDL-C levels decreased[35]. PCSK9 has been shown to be an independent predictor of increased c-IMT independently of classical factors including Lp(a) concentration in asymptomatic adults[29]. Study by Toth et al.[30] showed that PCSK9 was an independent predictive factor not only for increased c-IMT, but also for another morphological feature of the arterial vessel wall, the PWV. On the other hand, treatment with PCSK9 inhibitors leads to improvements in all mentioned properties of the vessel wall, but the degree of improvement also depends on accompanying risk factors[31,32]. In our study, we found no association of PCSK9 concentration with FMD, c-IMT and PWV. This result is surprising only at a first glance. Due to genetic and lifelong exposure to increased LDL-C levels, our patients naturally had a negative impact on the properties of the arterial vessel wall. On the other hand, all patients were treated with statins for at least 6 months, which improved the properties of the arterial vessel wall, but to a lesser extent as they have been affected by lifelong exposure to elevated LDL-C levels. Statin treatment also significantly increases PCSK9 levels and thus disrupts this associations, which are otherwise present in patients without prior statin treatment[29,30].

Certainly, we cannot ignore that all our patients had greatly increased Lp(a) values, which independently of any other risk factors affects the properties of the arterial vessel wall[36,37].

Parameter	Sum of risk alleles		*p* value
	5–7	8–9	
Age (years)	49.4 ± 8.6	53.0 ± 8.2	0.080
Body mass index (kg/m^2)	28.3 ± 3.6	28.9 ± 4.2	0.540
Total cholesterol (mmol/L)	4.15 ± 0.73	4.34 ± 0.91	0.349
HDL cholesterol (mmol/L)	1.25 ± 0.33	1.15 ± 0.23	0.181
LDL cholesterol (mmol/L)	2.26 ± 0.62	2.37 ± 0.77	0.519
Triglycerides (mmol/L)	**1.44 ± 0.51**	**1.85 ± 0.96**	**0.032**
Lipoprotein (a) (mg/L)	1431 (11207–1783)	1431 (11207–1783)	0.409
Apolipoprotein B (g/L)	0.79 ± 0.22	0.82 ± 0.22	0.559
Apolipoprotein A1 (g/L)	1.37 ± 0.22	1.30 ± 0.14	0.133
Total PCSK9 (ng/mL)	264.3 (185.4–413.5)	321.3 (244.9–376.0)	0.801
FMD (%)	10.8 ± 6.0	11.0 ± 6.9	0.917
Beta stiffness	5.7 ± 1.1	5.9 ± 1.6	0.530
PWV β (m/s)	5.2 ± 0.7	5.4 ± 0.8	0.436
c-IMT (mm)	0.65 ± 0.12	0.65 ± 0.10	0.859

Table 4. Clinical, biochemical and arterial wall properties in groups with high and low numbers of *PCSK9* risk alleles. HDL, high-density lipoprotein; LDL, low-density lipoprotein; FMD, flow-mediated dilatation; PWV, pulse wave velocity; c-IMT, intima-media thickness. Values are means ± standard deviations or medians (lower–upper quartile). Sum of risk alleles was calculated from scores in Table 2. Student's t test or Mann-Whitney was used to test the differences between the groups. *p* values < 0.05 were considered as statistically significant. Significant values are in [bold].

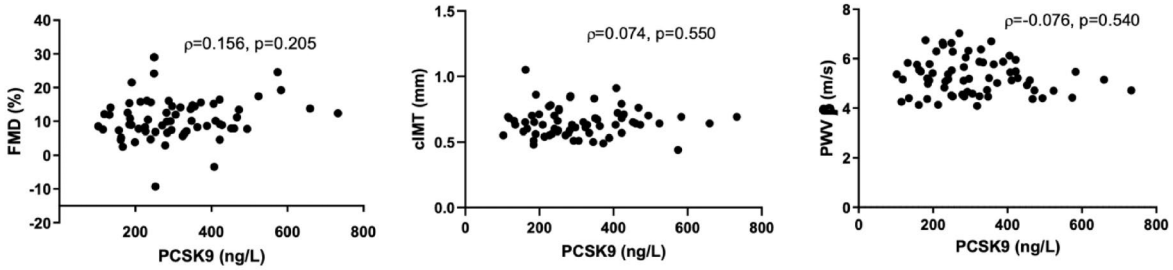

Fig. 3. Correlation between arterial wall properties and serum concentrations of total PCSK9. Spearman correlation analysis was performed (ρ, Spearman correlation coefficient).

Among the polymorphisms studied in our research, carriers of the risk alleles for rs11206510, rs11591147 and rs2479409 had increased values of both, Lp(a) and PCSK9[38]. One of the reasons for the increased value of Lp(a) in patients with *PCSK9* polymorphisms could be a decrease in the clearance of Lp(a). Namely, it turned out that the clearance of Lp(a) in hepatocytes of PCSK9-/- mice was found to be more than 50% higher than in wild-type hepatocytes[39]. Given the inclusion criteria for our research, it is not surprising that we were unable to detect these differences.

There are several limitations of our study. The main limitation of our study is the lack of the control group. However, we must be aware that the proper control group would need to include the patients with more or less the same characteristics (i.e. young patients in a stable phase after myocardial infarction and high Lp(a) values) without statin treatment. The inclusion of such a control group would be unethical. The best possible control group would present similar patients without elevated Lp(a) levels. Since our study lacks such control group, we cannot draw the conclusions among a wider range of Lp(a) levels. Thus, the conclusions of our study can only be applied to this specific group of patients, who are also at a highest risk for future cardiovascular events. Another limitation of our study is a relatively low number of patients. Assuming an alpha level of 0.05, an additive inheritance model, an assumed prevalence of disease of 30%, a power of the study of 80%, minor allele frequencies between 0.05% (rs1711503) and 36% (rs2479409), and genetic relative risks between 1.1 and 1.3 (which can be expected in genetic association studies on complex diseases such as CAD), the number of patients in our study should be larger. However, this is a consequence of a strict inclusion criteria that resulted in a relatively homogenous cohort of high-risk patient for future cardiovascular events.

Conclusions

The results of our study suggest that the 5 SNPs (rs11206510, rs2479409, rs2479408, rs1711503 and rs11591147) that were selected based on the reported evidence of their association with coronary artery disease, are not associated with lipid parameters nor with the properties of the arterial vessel wall in our cohort of high risk post myocardial infarction patients treated with maximum tolerated dose of statins and severely increased Lp(a) values. The most likely explanation for this result might be statin treatment, which, by affecting both, PCSK9 and arterial vessel wall properties, disrupts the correlations between these parameters. Another explanation could be severely increased Lp(a) values in these patients, which increase even further during statin treatment. In order to clarify this dilemma, it would be of great interest to include a group of patients with the same characteristics but normal Lp(a) values or a group of patients with the same characteristics but without statin treatment. Unfortunately, one cannot expect to be able to include a large group of patients after myocardial infarction who are not treated with statins. However, we must consider that patients with polymorphisms in the *PCSK9* gene are exposed to increased levels of both, LDL-C and Lp(a) from birth onwards, so atherosclerotic changes are present early in life, usually long before patients start with lipid-lowering treatment. Lipid-lowering treatment with statins as well as PCSK9 inhibitors or drugs specific for lowering Lp(a) slow down the atherosclerotic process, but they cannot completely eliminate the atherosclerotic changes that occurred before starting the treatment.

Data availability

The data presented in this study are available upon reasonable request from the corresponding author.

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Author contributions

Conceptualization and methodology, A.R.L. S.U., and M.Š.; investigation, A.R.L., M.Š., T.L., S.U. and J.Z.; resources, T.L., A.R.L., M.Š., S.U., J.Z., S.A. and K.T.P.; data curation, A.R.L, T.L., S.U. and M.Š.; writing—original draft preparation, M.Š., S.U, J.Z.; writing—review and editing, S.U. A.R.L., M.Š, T.L., J.Z., S.A., and K.T.P.; visualization, S.U. and J.Z.; supervision, M.Š.; project administration, M.Š.; funding acquisition, M.Š. and K.T.P. All authors have read and agreed to the published version of the manuscript.

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Declarations

Competing interests

The authors declare no competing interests.

Ethical approval

The study was conducted in accordance with the Declaration of Helsinki and approved by the Slovenian Ethics Committee for Research in Medicine (KME 0120-357/2018/8; date of approval: 23 April 2019 and KME 0120-317/2021/3; date of approval: 2 August 2021).

Consent to participate

Informed consent was obtained from all subjects involved in the study.

Additional information

Correspondence and requests for materials should be addressed to S.U.

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