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Treatment with PCSK9 inhibitors influences microRNAs expression and changes of arterial wall properties: a randomized controlled trial

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Abstract

Background MicroRNAs (miRNAs) are involved in the synthesis of proprotein convertase subtilisin–kexin type 9 (PCSK9), one of the regulators of low-density lipoprotein cholesterol (LDL-C) metabolism, and are directly involved in the atherosclerotic process. The aim of this study was to verify whether treatment with PCSK9 inhibitors (PCSK9i) and changes in the expression of miRNAs involved in PCSK9 metabolism are associated with arterial wall properties in stable post-myocardial infarction (MI) patients with insufficiently regulated LDL-C levels and significantly increased Lp(a) levels.

Methods Ninety-five patients after MI were enrolled and randomized to a placebo ($N=31$) or PCSK9i group ($N=64$). The treatment group received subcutaneous alirocumab 150 mg or evolocumab 140 mg, every 2 weeks. Blood for biochemical and epigenetic analysis was taken and ultrasound measurements of flow-mediated dilation of brachial artery (FMD), carotid intima–media thickness (c-IMT) and pulse wave velocity (PWV) were performed initially and after 6 months of treatment. The expression of the selected 5 miRNAs (miR-191-5p, miR-224-5p, miR-337-3p, miR-483-5p, and miR-552-3p) was quantified using quantitative polymerase chain reaction.

Results A decrease in c-IMT was associated with a decrease in the expression of miR-337-3p ($\rho=0.329$; $p=0.010$) and miR-483-5p ($\rho=0.324$; $p=0.012$). We did not detect any associations between miRNA changes and FMD or PWV.

Conclusions Our results suggest that changes in the selected miRNAs are associated with changes in the morphological properties of the arterial wall. We have shown that the decrease in miR-483-5p expression present a good indicator of the regression of morphological atherosclerotic change.

The trial registration: The study is registered with CinicalTrials under the number NCT04613167, date of registration November 2nd, 2020. Approval for this study was obtained from the National Medical Ethics Committee of the Republic of Slovenia (reference number: KME 0120-357/2018/8).

Keywords MiRNA, Endothelial function, PCSK9 inhibitors, Myocardial infarction, Low-density lipoprotein cholesterol

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Background

Treatment with proprotein convertase subtilisin–kexin type 9 inhibitors (PCSK9i) was shown to be effective not only in lowering low-density lipoprotein cholesterol (LDL-C), but also in reducing the cardiovascular morbidity and mortality [1, 2]. Treatment with alirocumab reduced the risk of major adverse cardiac events (MACE) in patients after a recent acute coronary syndrome with LDL-C close to 1.8 mmol/L and lipoprotein(a) (Lp(a)) values above 137 mg/L. In contrary, such an effect was not observed in patients with Lp(a) values below 137 mg/L. In patients with higher LDL-C levels, the MACE risk decreased regardless of the Lp(a) levels [3]. Most likely all of the positive effects of PCSK9i could not be attributed only to the reduction of LDL-C and Lp(a). Part of the so-called pleiotropic effects of PCSK9i is also related to factors affecting PCSK9 metabolism. In recent years, an increasing attention has been attributed to non-coding RNAs, among which microRNAs (miRNAs) play an integral role. The main function of miRNAs is to suppress the synthesis of a specific target protein. The miRNAs are also involved in the synthesis of PCSK9, one of the main regulators of the LDL-C metabolism. It was previously shown that miR-191-5p, miR-224-5p, miR-337-3p, and miR-483-5p are involved in the regulation of PCSK9 concentrations. The expression of miR-191-5p and miR-224-5p were inversely proportional to PCSK9 concentration in cell cultures [4]. In addition, an increased expression of miR-224-5p was shown to increase the LDL receptor (LDLR) expression on the surface of the hepatocytes and increase the LDL-C binding [5]. In the livers of diet-induced obesity [high-fat-diet (HFD)-fed] mice, the expression of miR-337-3p was significantly lower than in controls, while the concentration of LDL-C positively correlated with the expression of miR-337-3p [6]. Circulating miR-483-5p levels were decreased in the patients with elevated LDL-C levels, while significant reductions in both, total and LDL-C were observed in HFD-fed mice with miR-483-5p supplementation [7].

However, miRNAs are not only involved in the LDL-C metabolism, but also directly in the atherosclerotic process itself. They are involved in the foam cell formation, which is the first step leading to endothelial dysfunction. Decreased expression of miR-224-5p was present in both, plasma, and atherosclerotic plaques in HFD-fed mice. Decreased expression of miR-224-5p leads to accelerated oxidation of LDL-C, leading to endothelial cell damage and apoptosis [8]. Thus, miR-224-5p could serve as a marker of LDL-C oxidation and an interesting target for atherosclerotic treatment. In addition to increasing LDL-C, increased expression of miR-155 also promotes the formation of the atherosclerotic plaques as shown by Peng et al. [9]. At the same time, the inhibition of miR-155 in HFD-fed mice led to a reduction in both, LDL-C concentration, and the atherosclerotic plaque formation. In addition to the direct effect on the LDL-C concentration, the reduction of miR-155 expression on the formation of the atherosclerotic plaques could also affect the production of the pro-inflammatory cytokines, in particular interleukin (IL)−18 and IL-1ß [9]. miR-206 is also involved in the development of carotid atherosclerosis. Its concentration was significantly lower in patients with asymptomatic carotid atherosclerosis compared to their healthy peers [10]. The expression of miR-206 was negatively associated with the degree of carotid artery stenosis in patients, while lower miR-206 expression was associated with a higher risk of future cardiovascular events over a 5-year period [10].

In this study, we included the patients in the stable phase after myocardial infarction (MI) who, despite receiving the maximum tolerated statin dose and ezetimibe if needed, did not achieve sufficiently controlled LDL-C values. In addition, all of them had significantly increased Lp(a) values. The patients were treated with PCSK9i, which are known to reduce LDL-C levels by 50–70% and Lp(a) levels by 20–40% [1, 2]. Improvement of the functional and morphological properties of the arterial vessel wall after treatment with PCSK9i was already demonstrated in different groups of patients [11–13]. We have also shown that treatment with PCSK9i affects the expression of miRNAs involved in the PCSK9 metabolism [14]. Improving the functional and morphological properties of the arterial vessel wall in patients with coronary artery disease significantly reduced the risk of future cardiovascular events [15]. However, whether the changes in the expression of miRNAs involved in the PCSK9 metabolism could be related to both, the properties of the arterial vessel wall and changes resultant on the treatment with PCSK9i, remains to be solved.

This study is a pre-specified sub-analysis of our previously published study [11]. The primary endpoint of our previously published study [11] was to investigate the impact of PCSK9i on the properties of the arterial vessel wall as well as on the lipid profile. The secondary endpoint of our previous study [11] was to investigate the epigenetic mechanisms behind the effects of the treatment with PCSK9i which are presented in the current study. Therefore, the purpose of our current research was to identify whether treatment with PCSK9i and the resulting changes in the expression of miRNAs involved in the PCSK9 metabolism are associated with the arterial wall properties and their changes in post-MI patients with insufficiently regulated LDL-C and highly elevated Lp(a) levels.

Methods

Patients

We included 95 patients in the stable phase of coronary artery disease at least 6 months after acute MI. All patients received optimal medical therapy that had not been changed for at least 2 months before enrolment in the study, including the maximum tolerated dose of statin and ezetimibe as needed and no other lipolytic drugs. In addition, all risk factors except lipids were adjusted according to current guidelines [16]. Patients with first acute coronary syndrome before the age of 55 and an Lp(a) level above 1000 mg/L or an Lp(a) level above 600 mg/L and an LDL-C level of more than 2.6 mmol/L were included. They were randomised into three groups regarding the treatment with placebo or PCSK9i. The first group was the placebo group which included 31 patients and received standard dyslipidaemia treatment for 6 months. The second and the third groups of 64 patients (32 in each group), received treatment with a PCSK9i, namely, alirocumab at a dose of 150 mg or evolocumab at a dose of 140 mg, every 2 weeks for 6 months. Given that there were no differences between the alirocumab and evolocumab group, these two groups were combined into one group (active treatment). Patients with elevated liver transaminases enzyme activity (more than three times above the reference values) and severe renal dysfunction (serum creatinine more than 200 µmol/L) were excluded. We also excluded those with acute illness in the last 6 weeks.

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) and K3 EDTA tube. The further biochemical method of sample analysis and the type of parameter determination have already been described in [17]. The Friedewald formula [18] was used to calculate LDL-C. EDTA tubes were centrifuged at 300×*g* for 10 min. Plasma was then transferred to a new tube and centrifuged again at 400×*g* for 5 min to obtain platelet-poor plasma.

RNA isolation and measurement of miRNAs expression

Isolation of RNA and determination of miRNA expression have been described in detail previously [14]. In summary, circulating plasma RNA was isolated using the NextPrep™ Magnazol™ cfRNA Isolation Kit (PerkinElmer, Waltham, MA, USA), and transcribed into complementary DNA (cDNA) using the miRCURY Locked Nucleic Acid (LNA) Universal RT Kit (Qiagen, Hilden, Germany). The expression of selected 6 miRNAs (miR-191-5p, miR-224-5p, miR-337-3p, miR-483-5p and miR-552-3p) was quantified by quantitative polymerase chain reaction (qPCR) using miRCURY LNA miRNA PCR Assays (Qiagen, Hilden, Germany). hsa-miR-16-5p and hsa-miR-4516 were used as reference miRNAs [19]. Relative expression was calculated as $2^{-\Delta Cq}$, where ΔCq = average Cq (target miRNA) – average Cq (reference miRNAs) [20].

Ultrasound measurements

Endothelial function, as an indicator of functional properties, was measured as brachial artery flow-mediated dilation (FMD). Morphological properties were determined by measuring arterial stiffness on the right common carotid artery (pulse wave velocity (PWV)) and the thickness of the intima–media complex in the far wall of carotid arteries (c-IMT). All measurements were performed as described in our previous study [21]. Briefly, FMD and PWV were measured using Aloka prosound α7 (Hitachi Aloka Medical, Ltd., Japan) equipped with special software with an integrated high-resolution eTracking system for automatic determination of endothelial parameters for subsequent changes in vessel wall diameter (Hitachi Aloka, Wallingford, CT, USA) and a 10 MHz linear array transducer. c-IMT measurements were performed of both sides of the common carotid artery using Vivid E95 ultrasound machine. The c-IMT was automatically calculated using the EchoPAC program, as the mean and standard deviation in the marked part (2 cm proximal to the bulb in the common carotid artery over 2 cm, and from the ostium of the internal carotid artery, over 1.5 cm) of the intima media of the carotid artery.

Statistical analysis

For descriptive statistical analysis, we analysed the normality of the distribution of continuous variables with the Shapiro–Wilk test. We used the median with interquartile range or the mean with standard deviation to describe continuous variables. Frequencies were used to describe the distribution of the categorical variables. The Chi-square test was used to compare the distribution of the categorical variables between different groups, whereas the *t* test or Mann–Whitney *U* test was used for the continuous variables. The Wilcoxon signed rank test was used to assess changes during the placebo and treatment periods. Correlations between continuous variables were calculated using Spearman's ρ coefficient. All statistical tests were two-sided. Multiple regression analysis was used to find the independent determinants for the variations in the properties of the arterial vessel wall. We used 0.05 as the level of statistical significance. For the miRNA analysis, we have considered a Bonferroni correction and reduced the level of significance to 0.01. IBM SPSS Statistics version 27.0 (IBM Corporation,

New York, NY, USA) was used to perform the statistical analyses. Figures were generated using GraphPad Prism 9 (San Diego, CA, USA). GPower was used to perform the power of the study calculations [22]. The required sample size as determined using the *apriori* analysis (with the 0.80 power of the study, 0.15 effect size and 0.05 α error) was 22 subjects per group.

Results

Baseline characteristics

At the beginning of the study, the group of patients who initially received placebo did not differ significantly from the group that received PCSK9i in any parameters (the anthropometric parameters, systolic and diastolic blood pressure, lipid parameters and total PCSK9). There were also no differences between the groups in the expression of the measured miRNAs, except for miR-191-5p, where the expression was higher in the placebo group (Table 1). There were also no differences between the groups in the functional and morphological properties of the arterial vessel wall (Fig. 1).

Effects of treatment with PCSK9i

The effects of PCSK9i and placebo treatment on lipid profile are presented in Table 2. The results of the effect of PCSK9i treatment on TG, ApoA, ApoB, Lp(a) and total PCSK9 were published previously [17]. There were no significant differences in TG, ApoB and Lp(a) levels after 6 months in the placebo group. In the PCSK9i treatment group TG decreased from 1.50 mmol/L (1.06–2.11 mmol/L) to 1.20 mmol/L (0.78–1.81 mmol/L); $p < 0.001$. ApoB levels decreased from 0.82 g/L (0.63–0.98 g/L) to 0.35 g/L (0.35–0.48 g/L); $p < 0.001$ in the PCSK9i-treated group and increased from 0.80 g/L (0.73–1.00 g/L) to 0.82 g/L (0.67–0.96 g/L), $p = 0.099$ in the placebo group. After 6 months of treatment ApoA1 levels increased from 1.30 g/L (1.20–1.46 g/L) to 1.35 g/L (1.22–1.55 g/L); $p = 0.001$, and from 1.23 g/L (1.16–1.34 g/L) to 1.27 g/L (1.20–1.43 g/L); $p = 0.002$ in the placebo group. Lp(a) levels decreased from 1416 mg/L (1201–1781 mg/L) to 1133 mg/L (820–1664 mg/L); $p < 0.001$ in the PCSK9i-treated group, and from 1491 mg/L (1185–1739 mg/L) to 1397 mg/L (1224–1574 mg/L); $p = 0.701$ in the placebo group. Levels of total PCSK9 increased in the PCSK9i-treated group from 285.7 ng/L (210.0–401.3 ng/L) to 2784.3 ng/L (2508.4–3185.8 ng/L); $p < 0.001$ and from 240.3 ng/L (195.1–364.4 ng/L) to 352.1 ng/L (251.1–469.0 ng/L); $p = 0.007$ in the placebo group.

After the treatment, there was a significant improvement in both, functional and morphological properties of the arterial vessel wall. FMD improved from 10.9 ± 6.3% to 14.4 ± 5.7% in the PCSK9i-treated group, and from 10.8 ± 6.8% to 11.2 ± 4.6% in the placebo group

Table 1 Clinical and biochemical variables at baseline

Parameter	PCSK9i group (*N* = 64)	Placebo group (*N* = 31)	*p*
Age (years)	51.7 ± 8.7	48.9 ± 9.6	0.186
Body mass index (kg/m^2)	28.7 ± 8.7	28.7 ± 3.9	0.914
Systolic blood pressure (mmHg)	128 ± 15	126 ± 9	0.376
Diastolic blood pressure (mmHg)	77 ± 9	77 ± 7	0.897
Total cholesterol (mmol/L)	4.24 ± 0.83	4.20 ± 0.76	0.723
HDL cholesterol (mmol/L)	1.20 ± 0.28	1.12 ± 0.24	0.306
LDL cholesterol (mmol/L)	2.35 ± 0.70	2.29 ± 0.71	0.856
Triglycerides (mmol/L)	1.65 ± 0.79	1.72 ± 0.91	0.840
Lipoprotein(a) (mg/L)	1431 (11,207–1783)	1439 (969–1746)	0.915
Apolipoprotein B (g/L)	0.81 ± 0.22	0.84 ± 0.21	0.565
Apolipoprotein A1 (g/L)	1.34 ± 0.19	1.27 ± 0.17	0.170
C-reactive protein (mg/L)	0.87 ± 0.08	0.84 ± 0.06	0.752
Total PCSK9 (ng/L)	240.3 (195.1–364.4)	285.7 (210.0–401.3)	0.265
miR-191-5p ($2^{-\Delta Cq}$)	20.80 (15.01–25.37)	26.10 (20.31–29.82)	**0.010**
miR-224-5p ($2^{-\Delta Cq}$)	2.30 (1.24–3.61)	2.37 (1.35–3.97)	0.665
miR-337-3p ($2^{-\Delta Cq}$)	0.85 (0.45–1.70)	0.95 (0.63–1.51)	0.503
miR-483-5p ($2^{-\Delta Cq}$)	0.015 (0.008–0.023)	0.014 (0.010–0.028)	0.068

Bold values are statistically significant

Data are medians (lower–upper quartile) or means ± standard deviation. The differences between the two groups were calculated with *t* test or Mann–Whitney test

HDL high-density lipoprotein, *LDL* low-density lipoprotein, *PCSK9* proprotein convertase subtilisin–kexin type 9, *PCSK9i* PCSK9 inhibitors

*$p < 0.01$

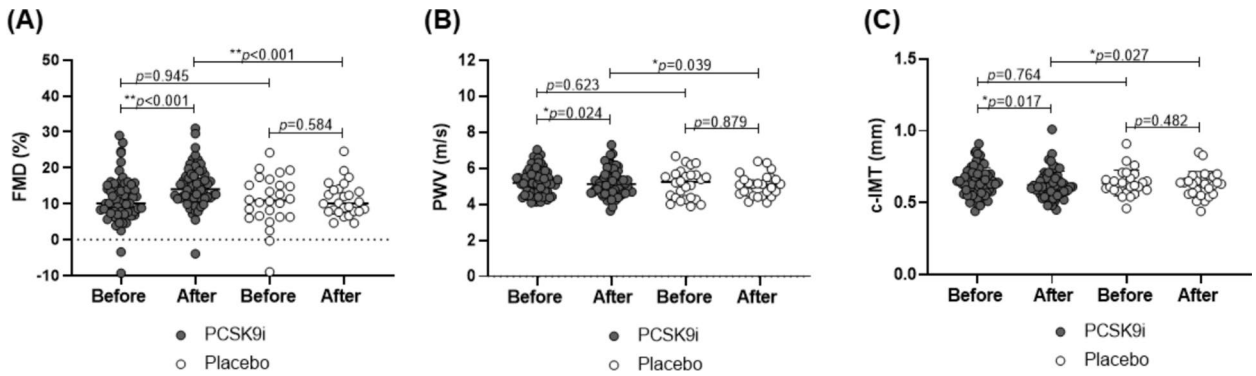

Fig. 1 Functional (**A**) and morphological (**B, C**) properties of the arterial vessel wall before and after treatment with PCSK9 inhibitors (PCSK9i).*$p < 0.05$, **$p < 0.001$

Table 2 Lipids at baseline and after 6 months of treatment

Parameter	Group	Baseline	After 6 months	p
Total-C	Placebo	4.31 ± 0.97	4.39 ± 0.94	0.431
(mmol/L)	PCSK9i	4.27 ± 0.83	2.71 ± 0.91	**** < 0.001**
LDL-C	Placebo	2.42 ± 0.97	2.43 ± 0.80	0.735
(mmol/L)	PCSK9i	2.32 ± 0.71	0.87 ± 0.80	**** < 0.001**
HDL-C	Placebo	1.12 ± 0.23	1.20 ± 0.30	0.010
(mmol/L)	PCSK9i	1.19 ± 0.27	1.26 ± 0.32	**** < 0.001**

Bold values are statistically significant

Data are medians (lower–upper quartile) or means ± standard deviation. The difference between the parameters at baseline and after 6 months of treatment within each group were calculated using paired samples *t* test or Wilcoxon matched-pairs signed-rank test

Total C total cholesterol, *LDL-C* low-density lipoprotein, *HDL-C* high-density lipoprotein

**$p < 0.001$

($p < 0.001$, $p = 0.584$, respectively). In the PCSK9i-treated group, PWV changed from 5.8 ± 1.4 m/s to 5.5 ± 1.6 m/s ($p = 0.024$), and in the placebo group from 5.6 ± 1.5 m/s to 5.5 ± 1.1 m/s ($p = 0.879$). The improvement was significantly higher in the PCSK9i-treated group ($p = 0.007$). c-IMT decreased in the PCSK9i-treated group (from 0.65 ± 0.11 mm to 0.63 ± 0.10 mm; $p = 0.017$), while there was no change in the placebo group (from 0.64 ± 0.09 mm to 0.63 ± 0.09 mm; $p = 0.482$) (Fig. 1).

Associations between biochemical parameters, expression of miRNAs and properties of the arterial vessel wall

At baseline, miR-191-5p and miR-224-5p expression were not associated with the concentrations of lipid parameters. The expression of miR-337-3p correlated borderline with the concentration of apoB ($\rho = -0.249$; $p = 0.017$). The expression of miR-483-5p correlated borderline with the concentration of HDL-C ($\rho = 0.258$; $p = 0.013$) and apoA1 ($\rho = 0.248$; $p = 0.018$). No correlation was found between the expression of any miRNA and the functional and morphological properties of the arterial vessel wall as well.

Upon PCSK9i treatment, the change in miR-191-5p expression correlated with the change in total PCSK9 concentration ($\rho = -0.429$; $p < 0.001$), and the change in the expression of miR-483-5p with the change in Lp(a) concentration ($\rho = -0.344$; $p = 0.007$). No other correlations between changes in miRNAs expression and lipid parameters were found.

A decrease in c-IMT was associated with a decrease in the expression of miR-337-3p ($\rho = 0.329$; $p = 0.010$) and miR-483-5p ($\rho = 0.367$; $p = 0.004$), while miR-224-5p showed borderline association ($\rho = 0.303$; $p = 0.019$) (Fig. 2). There were no associations between a decrease in c-IMT and changes in lipoproteins values. The importance of the variables that showed significance in the univariate analysis in predicting decrease of c-IMT after treatment with PCSK9i was tested in several linear regression models. Table 3 presents the conclusions of the best model which explained 76.4% of the variability in the decrease of c-IMT ($p = 0.015$). We did not find any other correlations between changes in the miRNA expressions, lipoprotein values and changes in the properties of the arterial vessel wall.

Discussion

The main finding of our research is that changes in the selected miRNAs involved in the metabolism of PCSK9, one of the most important regulators of LDL-C concentration, were associated with changes in the morphological properties of the arterial vessel wall. PCSK9i are the first drugs in clinical use that significantly decrease Lp(a) in addition to LDL-C. In the current study we included the patients after MI and insufficiently controlled LDL-C levels, despite the maximum tolerated statin dose. At the same time, these patients also possessed highly increased Lp(a) values. The treatment with PCSK9i showed

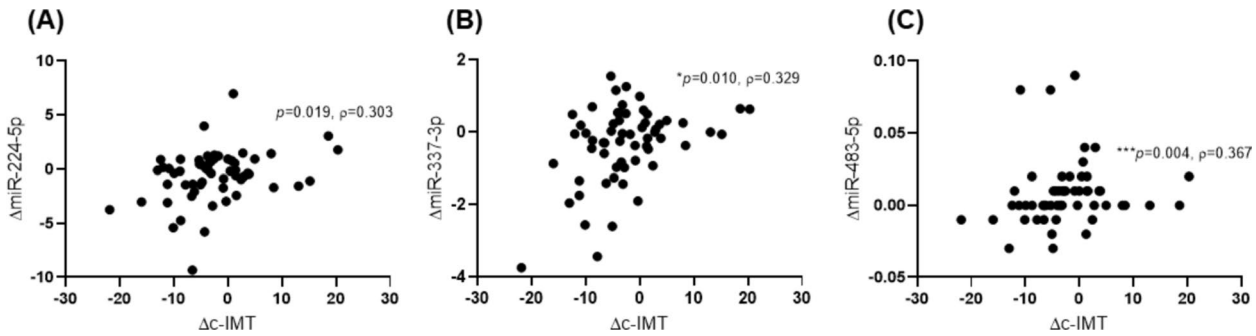

Fig. 2 Correlations between changes of miR-224-5p (**A**), miR-337-3p (**B**) and miR-483-5p (**C**) and c-IMT after treatment with PCSK9i. Spearman correlation analysis was used to calculate the correlation coefficient (ρ) and *p* value. **p* < 0.01, ***p* < 0.001

Table 3 Linear regression model of predictors of c-IMT decrease

Parameter	β	Tolerance	*p*
Age	− 0.076	− 0.612	*p* = 0.543
Δ Lipoprotein (a)	− 0.265	− 2.768	*p* = 0.236
Δ miR-224-5p	0.160	1.090	*p* = 0.281
Δ miR-483-5p	− 0.059	− 0.436	*p* = 0.665
Δ miR-337-3p	**1.075**	**0.360**	***p* = 0.024**

R^2 (part of variability explained with the model) = 0.764, *p* = 0.015; Changes (Δ) were obtained by calculating the differences between the value of the parameter after 6 months and at baseline. **p* < 0.05; values in bold are statistically significant

anticipated effect on lipid parameters as described in our previous study [14].

In the same patient cohort, we have previously demonstrated an improvement in both, the functional and morphological properties of the arterial vessel wall [11]. While the improvement in FMD was only associated with an increase in the ApoA1 concentration, a decrease in c-IMT was associated with a decrease in the atherogenic lipoproteins (total cholesterol, LDL-C and ApoB). We found no associations with the improvement in PWV. On the other hand, Silla et al. [23] found a significant improvement in PWV in high-risk patients 2 month post-evolocumab treatment. Moreover, the improvement in PWV was significantly higher in patients who had been previously treated with statins [23].

As we have previously reported, PCSK9i treatment significantly increased the expression of miR-191-5p and miR483-5p, while the increase in the expression of miR-224-5p and miR-337-3p was borderline statistically significant [14]. Considering that all these miRNAs are involved in the metabolism of PCSK9 and thus of LDL-C, it is obviously of interest to understand how their expression is affected by the statins that all our patients received. The data about this is scarce as only one study examined the effect of pitavastatin and atorvastatin on miR-483-5p expression [24]. Treatment with both statins increased the expression of miR-483-5p independently of changes in lipid parameters. This study suggests that miR-483-5p expression is an independent risk factor for future cardiovascular events and associated with multiple risk factors in the population without evident atherosclerotic disease. In this study a correlation between miR-483-5p and HDL-C concentration was found [25], while in this study, this correlation was only borderline significant Even though many miRNAs are involved in the metabolism and function of HDL-C, we do not have any data on the role of miR-483-5p [26, 27]. In more than 1200 apparently healthy subjects, the expression of miR-483-5p was shown to be a good predictive factor for the presence of atherosclerotic plaques in carotid arteries and c-IMT [28]. Despite all our patients were already receiving statins, PCSK9i treatment led to an increase in the miR-483-5p expression and a decrease in c-IMT, which were also significantly correlated with each other in this study. Our results are the first to show that the increase in the miR-483-5p expression can be a good indicator of the regression of morphological atherosclerotic changes. Given that the expression of miR-483-5p was associated with several risk factors (HDL-C, TG, and diabetes), which are related to both, the inflammation, and the coagulation-fibrinolytic process, it could be assumed that the regression of c-IMT is also a consequence of these changes.

However, the increase in the miR-483-5p expression was associated with a decrease in Lp(a) concentration. Hence, we it might be that in this case the PCSK9i showed lipid lowering effect. However, we must keep in mind that the primary action of PCSK9i is the reduction of LDL-C with a well-known mechanism, while the mechanism behind their reduction of Lp(a) is not fully elucidated. Some studies suggests that PCSK9i could act in two ways, i.e., enhancing the clearance of Lp(a) [29], and reducing its production [30].

Improvement in c-IMT was also associated with an increase in the expression of miR-337-3p. Of all five miRNAs studied in our research, miR-337-3p is the only one directly involved in the PCSK9 metabolism and thus also in the LDL-C metabolism. In HFD-fed murine models, the expression of miR-337-3p in the liver was significantly lower than in controls, while serum LDL-C concentration was inversely related to the expression of miR-337-3p [6]. The overexpression of miR-337-3p decreased the LDL-C concentration. The same study also showed that miR-337-3p only affects PCSK9 and no other pathways involved in the clearance of LDL-C as only PCSK9 gene expression decrease under the influence of miR-337-3p. This, of course, resulted in a consequent decrease of PCSK9 and LDL-C concentrations. Moreover, miR-337-3p overexpression only affected the concentration of LDL-C but not the concentration of HDL-C and TG [6]. However, there is no data on whether miR-337-3p overexpression affects the concentration of Lp(a), which contains many LDL-C-like particles that are equally, if not more, atherogenic than LDL-C itself.

miRNA-224-5p has been previously associated with reduced coronary flow reserve, reflecting impaired endothelial function in post-MI patients [31]. Unfortunately, this study does not include data on the influence of lipolytic therapy on miRNA's expression in their group of patients, so the direct comparison of the results is not feasible. The main characteristic of the patients in this study is that, in addition to moderately increased LDL-C, they also had significantly increased Lp(a) values, despite receiving the maximum tolerated dose of statin. Despite a significant reduction of Lp(a) values following PCSK9i treatment, at the end of the study the Lp(a) values in our patients were still significantly increased according to current recommendations. In this study, an increase in miR-224-5p expression showed only borderline association with improvement in c-IMT. Moreover, miR-224-5p was also not associated with the change in FMD as a measure of endothelial function, which was otherwise significantly improved. As might be expected, endothelial dysfunction is only a functional precursor of morphological changes in carotid arteries. In a previous study [5], Salerno et al. determined that the primary origin of miR-224-5p are hepatocytes, even though it functions in endothelial cells. Transport from hepatocytes to endothelial cells takes place in extracellular vesicles, which are important for the action of miR-224-5p as they found that miR-224-5p enclosed in extracellular vesicles has no effect on endothelial cells. Deficiency of miR-224-5p through activation of the transforming growth factor beta signalling pathway leads to a decreased metabolic activity and apoptosis [32].

The overexpression of miR-337-3p did not affect the expression of other miRNAs such as miR-191, miR-222, miR-224, miR-483-5p and miR-520d-5p that also modulate the PCSK9 metabolism. Given that all studied miRNAs in our research are involved in the PCSK9 metabolism, we anticipated the changes in their expressions following treatment with PCSK9i. As mentioned, Xu et al. reported that the overexpression of miR-337-3p does not affect the expression of the rest of the four miRNAs studied in our research [6]. However, they used animal models to show the influence of the overexpression of a particular miRNA on other miRNAs with no aim to evaluate the influence of the treatment. While the treatment with PCSK9i reduces the concentration of the free PCSK9 via binding of the antibodies to PCSK9, it does not affect the gene expression of PCSK9. Whether the PCSK9i exhibit only the reduction of LDL-C and Lp(a) levels, or also the additional mechanisms of action, a comparison between PCSK9i and siRNA, i.e., inclisiran could give us the answer [33]. Unfortunately, we were able to determine only the concentration of total PCSK9, hence we could not distinguish between free PCSK9 and PCSK9 bound to therapeutic antibodies. Only the expression of miR-191-5p negatively correlated with the change in total PCSK9 concentration in this study. miR-191-5p was also the only miRNA in this study that did not show statistically significant correlation with the changes in the arterial vessel wall properties. The negative association between miR-191-5p and total PCSK9 concentration seems surprising at a first glance, but it is probably due to the measurement of the concentration of total and not free PCSK9. Previous studies where concentration of the free PCSK9 was measured, showed that this concentration decreased significantly after PCSK9i treatment [34]. Studies like ours, where the concentration of total PCSK9 was measured, report similar results, i.e., the concentration of total PCSK9 increased significantly after PCSK9i treatment. Hence, both, free and total PCSK9 levels could serve as an indicator of the patient compliance [35]. The association between changes in c-IMT and the expression of miR-337-3p and miR-483-5p, with no associations with changes in lipoproteins and total PCSK9, could suggest the so-called pleiotropic effects, meaning the effects of PCSK9i beyond their primary effect on lipoproteins. PCSK9 interacts with specific surface receptors, contributing to cardiovascular disease through both LDLR- dependent and independent pathways. These receptors include members of LDLR superfamily, such as LDLR, very low-density lipoprotein receptor (VLDLR), low-density lipoprotein receptor-related protein 8 (APOER2), as well as other proteins and receptors like cyclase associated actin cytoskeleton regulatory protein 1

(CAP1), CD36, and ATP-binding cassette transporter A1 (ABCA1) [36].

There are at several shortcomings of this study. First, the relatively small number of subjects, which is a consequence of the very strict inclusion criteria, which can, on the other hand, be an advantage due to the very homogeneous population. Second, the lack of the free PCSK9 measurement after PCSK9i treatment due to the limited specificity of the ELISA assay. It would be interesting to evaluate the correlations between the change in the free PCSK9 and the miRNAs studied. Third**,** lack of comprehensive interpretation and validation of the results. This would require multicenter and multi-ethical studies. Fourth, lack of long-term follow-up on clinical outcomes such as MI recurrence. However, the current study is a preliminary study, and our results provide the basis on the involvement of the studied miRNA in high-risk patients treated with PCSK9i. To determine if the miRNA identified here could be used as biomarkers for clinical efficacy such as MI recurrence and mortality, further larger and long-term clinical studies are needed. Fifth, the lack of mechanisms underlying the miRNA modulation of PCSK9 and vessel biology. To elucidate these mechanisms, further in vitro functional studies are suggested.

Conclusions

To the best of our knowledge this study is the first to evaluate the association between miRNAs involved in the PCSK9 metabolism and arterial vessel wall properties. We found that, in addition to their known effects on lipoproteins, PCSK9i also improve the properties of the vascular wall. Furthermore, PCSK9i influence the miRNAs involved in the PCSK9 metabolism independently of their lipolytic effect. Our results suggest that miRNA changes present an indirect mechanism of action of PCSK9i affecting the changes in the properties of the arterial vessel wall. Whether these are the pleiotropic effects or semi-effective lipolytic effects due to insufficiently reduced Lp(a) values, remains elusive. Further studies where patients will receive drugs that specifically reduce Lp(a) are anticipated to provide the answer to this question.

Abbreviations

apoA1	apolipoprotein A1
apoB	apolipoprotein B100
c-IMT	Carotid intima–media thickness
FMD	Flow-mediated dilation of brachial artery
HDL-C	High-density lipoprotein cholesterol
HFD	High-fat-diet
IL	Interleukin
LDL-C	Low-density lipoprotein cholesterol
LDLR	LDL receptor
Lp(a)	Lipoprotein(a)
MACE	Major adverse cardiac events
MI	Myocardial infarction
miRNA	microRNA
PCSK9	Proprotein convertase subtilisin–kexin type 9
PCSK9i	Proprotein convertase subtilisin–kexin type 9 inhibitors
PWV	Pulse wave velocity
TC	Total cholesterol
TG	Triglycerides

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Author contributions

Conceptualization and methodology, A.R.L. and M.Š.; investigation, A.R.L., T.L. and T.K.; resources, T.L., A.R.L., M.Š., J.Z. and K.T.P.; data curation, A.R.L, T.L., T.K., J.Z. and M.Š.; writing—original draft preparation, M.Š. and A.R.L.; writing—review and editing, A.R.L., M.Š, T.L., T.K., J.Z., and K.T.P.; visualization, A.R.L. 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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Availability of data and materials

No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate

All the procedures performed in this study that involved human patients 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 (reference number: KME 0120-357/2018/8). The study is registered with CinicalTrials under the number NCT04613167. The initial aim was to include at least 70 patients to achieve the desired power of the study. However, we managed to include even more patients ($N=95$) and to collect the samples for the current analysis. All patients signed a written informed consent prior to inclusion in the study.

Consent for publication

"Not applicable".

Competing interests

The authors declare no competing interests.

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