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Smoking and diabetes attenuate beneficial effects of PSCK9 inhibitors on arterial wall properties in patients with very high lipoprotein (a) levels

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ABSTRACT

Background and aims: Elevated lipoprotein (a) (Lp(a)) and low-density lipoprotein cholesterol levels (LDL-C) are significant residual risk factors for cardiovascular events. Treatment with protein convertase subtilisin kexin type 9 (PCSK9) inhibitors reduces the levels of both. Less is known about effects of PCSK9 inhibitors on functional and morphological properties of the arterial wall. The aim of the present study was to determine whether other factors besides decreased LDL-C and Lp(a) are associated with functional (flow-mediated dilation [FMD]) and morphological (carotid intima-media thickness [c-IMT], pulse-wave velocity [PWV]) changes of the arterial wall properties in patients with coronary artery disease (CAD) treated with alirocumab and evolocumab.

Methods: One hundred patients with CAD after myocardial infarction before 55 years and with high Lp(a) were randomised to lipid-lowering therapies without PCSK9 inhibitors (control; N = 31), or with alirocumab 150 mg SC (N = 35) or evolocumab 140 mg SC (N = 34), every 2 weeks. All patients underwent blood sampling for biochemical analyses and ultrasound measurements for FMD, c-IMT and PWV.

Results: There were no significant changes in FMD for the control (10.7% ± 6.6%–11.1% ± 4.4%, p = 0.716) and alirocumab (10.7% ± 5.9%–11.2% ± 5.3%, p = 0.547) groups, while evolocumab promoted significant increase (11.2% ± 6.8%–14.1% ± 6.6%, p < 0.0001). Only in non-smokers and non-diabetics significant improvements in FMD (p < 0.0001) after treatment with PCSK9 inhibitors were observed.

Conclusion: These data show that for patients with CAD and high Lp(a) levels, beneficial effects of PCSK9 inhibitors on the arterial wall properties can be attenuated by specific risk factors, such as smoking and diabetes.

Introduction

Despite recent progress, atherosclerotic cardiovascular disease (ASCVD) is still a leading cause of death worldwide [1]. One of the most important risk factors is high levels of low-density lipoprotein-cholesterol (LDL-C), although other atherogenic lipoproteins levels, such as those of lipoprotein (a) (Lp(a)), can result in important additional risk [2]. Lowering LDL-C and LDL particle levels via mechanisms that increase LDL receptor activity reduces the risk of ASCVD [3]. High LDL-C levels are associated with a thinner fibrous cap, a large lipid pool, a wide lipid arc and the presence of macrophages, and thus the arterial walls are more prone to rupture. Coronary plaque regression can be achieved when LDL-C levels show >50% decreases through treatment with statins [4]. However, even when LDL-C levels are lowered using lipid-lowering therapies, significant residual risk remains for individuals with elevated Lp(a), which increases with increasing Lp(a) levels [5].

Plasma Lp(a) is a complex particle with a >90% genetic trait. It is composed of one LDL particle and apolipoprotein a (apo(a)), which is linked to the apolipoprotein B (apoB) of LDL [6]. It is largely controlled by genetic variants and is the strongest genetic risk factor for coronary artery disease (CAD) [7]. A genetic study using a

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Mendelian randomization approach demonstrated a strong relationship between Lp(a) levels and CVD risk. Patients who had smaller apo(a) levels but higher Lp(a) levels were associated with a two-fold greater risk of CVD compared with those with the large isoform [8,9]. Inhibition of cholesterol synthesis by statins does not decrease Lp(a) levels [10]; indeed, this might even increase Lp(a) levels [11]. The atherogenic propensity of Lp(a) arises because of its structure. Its LDL-like particle, which is the main part of Lp(a), has similar effects to those of LDL-C, and it is even more atherogenic because of its higher sensitivity to oxidation [12].

Endothelial dysfunction is an early manifestation of development and progression of ASCVD and an independent predictor of cardiovascular (CV) events [13]. Endothelial function improves in response to pharmacotherapy, such as with statins, angiotensin-converting enzyme inhibitors and protein convertase subtilisin kexin type 9 (PCSK9) inhibitors [14,15]. Normal endothelial function is mediated through the release of nitric oxide (NO) by endothelial cells and can be assessed non-invasively by measuring changes in arterial diameter and blood flow in response to endothelial stimuli. Flow-mediated dilation (FMD) is an accurate and non-invasive method to assess endothelial function and it can be used as a surrogate marker of atherosclerosis and a predictor of CV events [16,17]. Impaired FMD is followed by morphological changes to the arterial wall that can be measured as increased intima media thickness of the carotid artery (c-IMT) [18]. Another morphological change in the arterial wall is the pulse wave velocity (PWV) [19]. Both c-IMT and PWV have been shown to be independent predictors of future CV events [20].

Despite treatments with statins at the highest tolerated doses, plus ezetimibe if necessary, many of these patients still have LDL-C above target levels [21]. These two groups of drugs on the other hand have no significant effects on Lp(a) levels. The currently clinically available human monoclonal immunoglobulin G2 antibodies against PCSK9, alirocumab and evolocumab, bind specifically to human PCSK9 to inhibit its effects on LDL receptors, which results in reductions in LDL-C and Lp(a) [22,23]. In patients enrolled in the Further Cardiovascular Outcomes Research with PCSK9 Inhibition in Subjects with Elevated Risk (FOURIER) trial [24], higher baseline Lp(a) levels were independently associated with increased risk of major CV events, and evolocumab significantly reduced Lp(a) levels by approximately 27%. In the Evaluation of Cardiovascular Outcomes After an Acute Coronary Syndrome During Treatment With Alirocumab (ODYSSEY Outcomes) trial [25], alirocumab significantly reduced Lp(a) levels from baseline to week 24, by 23%–29%. These reductions in Lp(a) were not dependent on baseline levels of Lp(a) or LDL-C, and they followed a dose-dependent effect. Evolocumab lowers plasma Lp(a) levels by decreasing the production of Lp(a) particles. Evolocumab might also act to increase Lp(a) catabolism through marked up-regulation of the LDL receptor, which would lead to enhanced Lp(a) clearance [24]. Recent findings by Watts et al. [26] indicated that in statin-treated patients, inhibition of PCSK9 with alirocumab decreased plasma Lp(a) levels by increasing the catabolism of circulating Lp(a) particles, with no effects on the production of these particles. This might be a consequence of marked up-regulation of hepatic receptors for LDL and decreased competition between Lp(a) and LDL particles for clearance by this receptor. Similar to evolocumab, it has also been proposed that the LDL receptor can become an important route for Lp(a) catabolism following administration of PCSK9 inhibitors, due to an increase in the number of LDL receptors, combined with the very low levels of apoB [27].

The aim of the present study was to determine whether other factors besides decreased LDL-C and Lp(a) are associated with changes in functional and morphological characteristics of the arterial wall after treatment with alirocumab and evolocumab. The novelty of this study is that we evaluated these associations for a specific group of patients, i.e., those who showed stable CAD following premature myocardial infarction and who showed very high Lp(a) levels.

Material and methods

Patients

We included patients aged between 18 and 65 years with clinically stable CAD of at least 6 months after myocardial infarction. Only patients who had a myocardial infarction before the age of 55 years and showed serum Lp(a) levels of 1000 mg/L irrespective of LDL-C levels or showed serum Lp(a) levels >600 mg/L and LDL-C >2.6 mmol/L were eligible. All of the patients had been prescribed beta blockers and antiplatelet drugs and were taking angiotensin-converting enzyme inhibitors/angiotensin II receptor blockers and statins at the highest tolerated doses, along with ezetimibe where needed. Their therapies had not been changed for at least 8 weeks before entering the study.

The main exclusion criteria were: elevated liver transaminases by more than three times the normal levels; severe renal impairment and serum creatinine >200 μmol/L; or history of acute illness in the previous 6 weeks.

The treatment strategy with PCSK9 inhibitors in our study followed the 2019 ESC/EAS guidelines for the management of dyslipidaemias: Lipid modification to reduce cardiovascular risk was in according to these guidelines [28]. The study was designed as a single blind meaning that the investigator (ARL) who performed the clinical examination and the ultrasound measurements, was unaware of the assigned group of the patients. Patients were randomised to three groups: standard lipid-lowering therapy with no PCSK9 inhibitors (control), or alirocumab 150 mg SC or evolocumab 140 mg SC, every two weeks. Senior investigator (MŠ) enrolled all patients and performed the randomization using the online programme Research randomizer (www.randomizer.org) to generate the random allocation sequences. Both the laboratory and ultrasound examinations were repeated after 6 months of the treatments.

All the procedures 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). Written informed consent was obtained from all the patients prior to inclusion in the study.

Clinical examination

Systolic and diastolic blood pressures were measured in the sitting position after a minimum of 10 min rest, with the mean of three measurements recorded. Anthropometric parameters were recorded, and body mass index was calculated.

Biochemical analysis

The blood for laboratory analysis was collected in the morning after 12 h overnight fasting. Samples were drawn from the antecubital vein into vacuum 5 mL tubes containing a clot activator (Vacutubes; LT Burnik, Slovenia). Total cholesterol, high-density lipoprotein-cholesterol (HDL-C), triglycerides, apoA1 and apoB were determined in the fresh serum by standard colorimetric or immunologic assays on an automated biochemistry analyser (Fusion 5.1; Ortho-Clinical Diagnostics, USA). Lp(a) was determined on the same biochemistry analyser using the Denka reagent (Randox, UK), which contains apo(a) isoform-insensitive antibodies, and

therefore showed minimal apo(a) size-related bias. LDL-C was calculated according to the Friedewald formula [29].

Flow-mediated dilation of the brachial artery

Endothelial function was assessed using FMD of the brachial artery using Aloka prosound α7 ultrasound device (Hitachi Aloka Medical, Ltd., Japan) which was equipped with special software with an integrated high-resolution eTracking system for automatic determination of the endothelial parameters for the latter changes in the diameter of the vessel wall (Hitachi Aloka, Wallingford, CT, USA) and a 10 MHz linear array transducer, according to the guidelines [30]. Measurements for each patient were performed at the same time of the day, after a 10-min rest period in a quiet temperature-controlled room. The patients rested in a supine position with the right arm extended and immobilised with foam, supported at an angle of approximately 80° from the torso. Blood pressure was recorded with an automated sphygmomanometer (digital blood pressure system; Welch Allyn Speidel & Keller OSZ) on the contralateral arm. Another blood pressure cuff was placed around the right forearm. Brachial artery diameter was visualised 5 cm−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 the baseline brachial artery diameter (1 min), the forearm blood pressure cuff was inflated to at least 50 mmHg above the patient systolic blood pressure for 4 min, to produce arterial occlusion. After the occlusion period, the cuff was rapidly deflated, to induce reactive hyperaemia, and the brachial artery diameter was recorded for 3 min. At the end of the measurements, the machine automatically provided the values of baseline and maximal brachial diameter and FMD (percentage change from baseline diameter of the brachial artery during reactive hyperaemia). All the images were recorded and saved onto an external hard drive.

Arterial stiffness parameters assessment

All of the arterial stiffness measurements were performed on the right common carotid artery with a linear vascular probe (working frequency, 5−13 MHz), as described in our previous study [31]. Testing was performed with the patients lying comfortably in a supine position with head elevation of around 45° and side tilt of 30° to the left, and in a quiet room with an air temperature of 22 °C−24 °C. For automatic determination of the stiffness parameters of the common carotid artery through analysis of the pulse wave, the ultrasound machine (Aloka prosound α7; Hitachi Aloka Medical Ltd., Japan) was also equipped with special software with an integrated high-resolution eTracking system (Hitachi Aloka, Wallingford, CT, USA). The echo-tracker cursor-pair was placed onto the anterior and posterior walls of the common carotid artery, 1 cm−2 cm proximal to the carotid bulb. Pressure waveforms were obtained non-invasively using arterial diameter change waveforms automatically calibrated based on the systolic and diastolic blood pressures measured as described above. The carotid artery local stiffness (β-stiffness) and the local PWV were automatically calculated as means of six beats. The measurements were repeated six times, with the mean value recorded.

Intima media thickness

All of the intima media thickness measurements were performed on both sides for the common carotid artery and on both sides for the internal carotid artery, according to the guidelines [32]. Plaques in the bulbs were recorded descriptively, as whether plaques (plaque c-IMT >1.1 mm) were present in the bulbs or not. Testing was performed with the patients in a supine position and under the same conditions as describe above, using an ultrasound machine (Vivid E95). Measurements were performed of the common carotid artery 2 cm proximal to the bulb, over 2 cm, and from the ostium of the internal carotid artery, over 1.5 cm. The c-IMT was automatically calculated using the EchoPAC program, as the mean and standard deviation in the marked part of the intima media of the carotid artery.

All examinations were performed by single investigator, unaware of the subjects' classification into a particular group, the duration of their treatment and their clinical and laboratory characteristics. For the assessment of the reproducibility of measurements, 30 subjects were randomly selected for repeated vascular studies. The correlation coefficient between the absolute differences and the mean values of paired measurements was 0.93 ($p < 0.05$). The reproducibility coefficient between investigators in our laboratory is 0.89 ($p < 0.05$).

Statistical analysis

Kolmogorov−Smirnov tests were used to define variables showing normal distributions, with these data expressed as means ± standard deviations. The non-normally distributed variables are expressed as medians and range (lower and upper quartiles). Pearson and Spearman correlation analysis was performed to determine the correlation of ultrasound parameters and lipid risk factors. General linear model analyses using delta values of the lipids (LDL-C, Lp(a), apoB) as covariates were performed to test the influence of the treatment and risk factors (smoking and diabetes) on the delta values of the vascular parameters. The differences between the three groups were calculated with one-way ANOVA or Kruskal-Wallis test for non-normally distributed variables. The differences in parameters between the treated and the placebo groups were compared using the delta values of the parameters (values at 6 months - values at baseline). The difference between the parameters at baseline and after 6 months of treatment were calculated using paired samples *t*-test. The differences between the two groups of patients with specific risk factor were calculated using Student's *t*-test. P values < 0.05 or adequately lower in the case of multiple comparisons, were considered statistically significant. Statistical analysis was performed using IBM SPSS Statistics for Windows (Version 25.0. Armonk, NY: IBM Corp.), and GraphPad Prism version 8 for Windows (GraphPad Software, San Diego, CA, USA; www.graphpad.com). GPower was used to perform the power of the study calculations [33]. The required sample size was determined using the *apriori* analysis with the 0.80 power of the study, 0.15 effect size and 0.05 α error probability.

Results

Patient characteristics

The 100 patients were recruited in this study during the period of November 2020 to May 2021 and followed for 6 months. All included patients showed clinically stable CAD of at least 6 months after myocardial infarction, with mean age at first coronary event <55 years. The patients were randomised to the control (N = 31), alirocumab (N = 35) and evolocumab (N = 34) groups. The mean ages of the groups showed borderline significant differences (47.5 ± 9.5, 52.8 ± 8.2, 49.9 ± 8.9 years, respectively; p = 0.060). There were no significant differences between the three groups for systolic (124 ± 10, 127 ± 15, 129 ± 15 mmHg, respectively; p = 0.434) and diastolic (76 ± 8, 77 ± 7, 77 ± 9 mmHg, respectively; p = 0.919) blood pressures (Table 1). There were 3 (9.7%), 9 (25.7%) and 4 (11.8%) current smokers in the respective groups; the

distribution of these smokers was not statistically significant between the groups (chi-squared test, p = 0.147). For diabetes mellitus type II, there were 7 (20.0%), 6 (17.1%) and 1 (2.9%) patients across the groups, respectively; the differences between the groups here were of borderline significance (chi-squared test, p = 0.090). The numbers of patients who were either current smokers or who had diabetes mellitus type II were 6 (19.4%), 12 (34.3%) and 4 (11.8%), respectively; the differences between the groups here were of borderline significance (chi-squared test, p = 0.071). When considering only the patients with active treatment (i.e., those in the alirocumab and evolocumab groups), there was no significance difference for current smoking (chi-squared test, p = 0.120), while the difference for diabetes mellitus type II was significant (chi-squared test, p = 0.030). Significant results were also achieved for the patients with either current smoking or diabetes mellitus type II in the alirocumab and evolocumab groups (chi-squared test, p = 0.026).

All the patients were treated with statins at the highest tolerated doses with or without ezetimibe, and all were treated with angiotensin-converting enzyme inhibitors, beta blockers and acetylsalicylic acid. One patient in each group received a calcium channel blocker. One patient in the alirocumab group did not finish the study due to problems associated with COVID-19 disease.

Lipid parameters

There were no differences between the three treatment groups at baseline for the serum lipid and lipoprotein levels (Table 1). After 6 months of treatment with alirocumab and evolucomab, within each of these active treatments there were significant decreases in total cholesterol, LDL-C, triglycerides, Lp(a) and apoB (Table 2). When compared to the control group, these patients on the active treatments showed significantly greater reductions in total cholesterol, LDL-C and apoB; instead, the reductions in Lp(a) levels for the active treatments showed borderline significance compared to the control group (p = 0.094) (Table 2). On the other hand, for all the treatment groups there were significant increases in apoA1, with HDL-C also significantly increasing, except for the alirocumab group (Table 2).

The relative changes at 6 months across all the lipids and lipoproteins for the three treatment groups are presented in Supplementary Fig. 1. Here it can be seen that total cholesterol increased in the control group by 3%, with alirocumab and evolocumab showing significant decreases of 34% and 36%, respectively (p < 0.0001). Similarly, LDL-C increased in the control group by 4% and significantly decreased with alirocumab and evolocumab by 64% and 63%, respectively (p < 0.0001). Small increases were seen for HDL-C in all treatment groups, although these showed no significant differences from the control group for the active treatments (7% vs. 5% vs. 8%, respectively; p = 0.725). For the triglycerides, there was an increase in the control group of 11%, compared to significant decreases with alirocumab and evolocumab of 11% and 17%, respectively (p = 0.005); here, however, there was no significant difference between the two active treatment groups (p = 0.817). For Lp(a) and apoB, the changes seen were again similar to those for total cholesterol. Lp(a) increased in the control group by 2%, with significant decreases for alirocumab and evolocumab of 17% and 23%, respectively (p < 0.0001), while apoB increased in the control group by 0.5% and significantly decreased for alirocumab and evolocumab by 40% and 45%, respectively (p < 0.0001). Across the active treatment groups here for both Lp(a) and apoB there were no significant differences (p = 0.748, p = 0.669, respectively). ApoA1 increased in the control group by 5%, and in both active treatment groups by 4%, with no significant differences seen here (p = 0.958).

For all these lipid parameters, when looking at the subgroups of patients with diabetes *versus* without diabetes and as current smokers *versus* non-smokers, there were no significant differences in the baseline and endpoint (i.e., 6 months of treatment) measures regardless of the treatment group (data not shown).

Vascular studies

For the vascular analysis, there were no significant differences in FMD, c-IMT and PWV between the three treatment groups at baseline (Table 3). For the functional (FMD) and morphological (c-IMT) properties of the arterial wall and the PWV, significant improvements were only seen for treatment with evolocumab (p < 0.0001, p < 0.0001, p = 0.004, respectively) (Table 3). However, significance was maintained across the treatment groups only for FMD (p = 0.044). Comparing the delta values (values at 6 months - values at baseline) between three treatment groups significant differences were found for FMD (p = 0.006) and c-IMT (p = 0.049), while PWV showed no significant difference (p = 0.061). Similarly, comparing the delta values (values at 6 months - values at baseline) between the placebo and treatment groups, significant differences were found for FMD (p = 0.013) and c-IMT (p = 0.037), while PWV showed no significant difference (p = 0.684).

For current smokers *versus* non-smokers, the improvement in FMD provided in active treatment groups, i.e. combined groups of alirocumab and evolocumab, reached significance for the non-smokers (p = 0.008), even though these non-smokers already

Table 1
Patient baseline clinical and biochemical parameters.

Parameter	Unit	Treatment group			p
		Control (N = 31)	Alirocumab (N = 35)	Evolocumab (N = 34)	
Age	years	47.5 ± 9.5	52.8 ± 8.2	49.9 ± 8.9	0.060
Systolic blood pressure	mmHg	124 ± 10	127 ± 15	129 ± 15	0.434
Diastolic blood pressure	mmHg	76 ± 8	77 ± 7	77 ± 9	0.919
Heart rate	beats/min	63 ± 11	62 ± 11	63 ± 8	0.960
Total cholesterol	mmol/L	4.3 ± 1.0	4.2 ± 0.9	4.4 ± 0.9	0.827
HDL-C	mmol/L	1.1 ± 0.2	1.2 ± 0.3	1.2 ± 0.3	0.366
LDL-C	mmol/L	2.4 ± 0.9	2.3 ± 0.7	2.4 ± 0.8	0.760
Triglycerides	mmol/L	1.7 ± 0.9	1.7 ± 0.9	1.6 ± 0.7	0.927
Lipoprotein (a)	mg/L	1491 (1185−1739)	1445 (1213−1791)	1372 (1021−1608)	0.684
Apolipoprotein B	g/L	0.86 ± 0.23	0.80 ± 0.23	0.84 ± 0.24	0.532
Apolipoprotein A1	g/L	1.27 ± 0.16	1.33 ± 0.19	1.35 ± 0.18	0.233

Data are means ± standard deviation, except for lipoprotein (a), where data are medians (lower-upper quartiles). The differences between the three groups were calculated with one-way ANOVA. For lipoprotein (a), the differences were calculated using Kruskal-Wallis test. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 2
Patient clinical and biochemical parameters at baseline and after 6 months of treatments.

Parameter	Unit	Group	Baseline	After 6 months	p
Total cholesterol	mmol/L	Control	4.3 ± 1.0	4.4 ± 0.9	0.425
		Alirocumab	4.1 ± 0.8	2.7 ± 0.8	**<0.0001**
		Evolocumab	4.4 ± 0.9	2.8 ± 1.1	**<0.0001**
		p	0.606	**<0.0001**	
HDL-C	mmol/L	Control	1.1 ± 0.2	1.2 ± 0.3	**0.010**
		Alirocumab	1.2 ± 0.3	1.2 ± 0.3	0.125
		Evolocumab	1.2 ± 0.3	1.3 ± 0.4	**0.001**
		p	0.369	0.379	
LDL-C	mmol/L	Control	2.4 ± 0.9	2.4 ± 0.8	0.735
		Alirocumab	2.2 ± 0.6	0.8 ± 0.6	**<0.0001**
		Evolocumab	2.4 ± 0.8	0.9 ± 0.9	**<0.0001**
		p	0.499	**<0.0001**	
Triglycerides	mmol/L	Control	1.7 ± 0.9	1.7 ± 0.8	0.920
		Alirocumab	1.7 ± 0.9	1.4 ± 1.0	0.037
		Evolocumab	1.6 ± 0.7	1.4 ± 0.7	**0.001**
		p	0.941	0.210	
Lipoprotein (a)	mg/L	Control	1491 (1185–1739)	1397 (1224–1574)	0.687
		Alirocumab	1445 (1231–1793)	1219 (845–1709)	**<0.0001**
		Evolocumab	1372 (1021–1608)	881 (800–1433)	**<0.0001**
		p	0.684	**0.033**	
Apolipoprotein B	mg/L	Control	0.86 ± 0.23	0.84 ± 0.26	0.815
		Alirocumab	0.80 ± 0.23	0.46 ± 0.18	**<0.0001**
		Evolocumab	0.84 ± 0.24	0.46 ± 0.24	**<0.0001**
		p	0.292	**<0.0001**	
Apolipoprotein A1	mg/L	Control	1.27 ± 0.16	1.33 ± 0.19	**0.001**
		Alirocumab	1.33 ± 0.19	1.38 ± 0.20	0.019
		Evolocumab	1.35 ± 0.18	1.39 ± 0.19	0.028
		p	0.238	0.314	

Data are means ± standard deviation, except for lipoprotein (a), as median (lower-upper quartile). The differences between the three groups were calculated with one-way ANOVA. For lipoprotein (a), the differences were calculated using Kruskal-Wallis test. The difference between the parameters at baseline and after 6 months of treatment within each group were calculated using paired samples *t*-test. HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol.

Table 3
Functional and morphological properties of the arterial wall at baseline and after 6 months of treatment.

Parameter	Unit	Group	Baseline	After 6 months	p
Flow-mediated dilation (Brachial artery)	%	Control	10.7 ± 6.6	11.1 ± 4.4	0.716
		Alirocumab	10.7 ± 5.9	11.2 ± 5.3	0.547
		Evolocumab	11.2 ± 6.8	14.1 ± 6.6	**<0.0001**
		p	0.945	**0.044**	
Carotid intima-media thickness	mm	Control	0.63 ± 0.09	0.63 ± 0.09	0.364
		Alirocumab	0.65 ± 0.10	0.64 ± 0.10	0.178
		Evolocumab	0.64 ± 0.11	0.62 ± 0.10	**<0.0001**
		p	0.764	0.576	
Pulse wave velocity	m/s	Control	5.1 ± 0.8	5.0 ± 0.7	0.554
		Alirocumab	5.1 ± 0.7	5.2 ± 0.8	0.515
		Evolocumab	5.5 ± 0.7	5.2 ± 0.7	**0.004**
		p	0.062	0.596	

Data are means ± standard deviation. The differences between the three groups were calculated with one-way ANOVA. The difference between the parameters at baseline and after 6 months of treatment within each group were calculated using paired samples *t*-test.

showed significantly greater FMD at baseline ($p = 0.011$); this benefit for the non-smokers remained significant after 6 months ($p = 0.006$) (Table 4). Both current smokers and non-smokers showed significant decreases in c-IMT with evolocumab, which provided significantly greater benefit for the non-smokers ($p = 0.016$). For diabetic *versus* non-diabetic patients, all of the baseline values of FMD, c-IMT and PWV were similar, as also for the changes seen following the treatments (data not shown). However, considering the combination of current smoking and diabetes with evolocumab treatment, significance was reached for the improvements to FMD ($p = 0.003$) only for the patients who were not current smokers and did not have diabetes (Table 5). General liner model that included LDL-C, Lp(a), treatment and smoking and diabetes explained 47% of the variability in FMD ($p < 0.0001$). The predictors that showed significant influence on FMD in this model were LDL ($p = 0.048$), treatment ($p < 0.0001$), smoking and diabetes ($p < 0.0001$) and the interaction between treatment and smoking and diabetes ($p < 0.0001$), indicating that the influence of treatment on FMD is less beneficial in patients that are smokers and diabetics in comparison with those that do not have these risk factors. The influence of Lp(a) in this model was not significant ($p = 0.422$).

Spearman correlation analysis between arterial wall properties and lipid and lipoproteins changes showed no significant results in patients treated with alirocumab. However, in the evolocumab group, the increase seen for FMD was significantly correlated with the increase in apoA1 ($r = 0.409$; $p = 0.020$). The decrease in c-IMT was significantly correlated with the decreases in total cholesterol ($r = 425$; $p = 0.014$), LDL-C ($r = 0.509$; $p = 0.02$) and apoB ($r = 0.393$; $p = 0.029$). No correlations were found between the changes in the lipids and lipoproteins and PWV. Finally, only the evolocumab group showed correlation between the functional and

Table 4
Functional and morphological properties of the arterial wall at baseline and after 6 months of active treatment of current smokers *versus* non-smokers.

Parameter	Unit	Group (N)	Baseline	After 6 months of active treatment	p
Flow-mediated dilation (Brachial artery)	%	Current smokers (6)	4.7 ± 7.6	6.2 ± 6.8	0.250
		Non-smokers (63)	11.6 ± 6.0	13.3 ± 5.7	**0.008**
		p	**0.011**	**0.006**	
Carotid intima-media thickness	mm	Current smokers (6)	0.70 ± 0.20	0.65 ± 0.16	0.042
		Non-smokers (63)	0.64 ± 0.10	0.61 ± 0.09	**<0.0001**
		p	0.290	0.156	
Pulse wave velocity	m/s	Current smokers (6)	5.50 ± 0.59	5.08 ± 1.05	0.291
		Non-smokers (63)	5.30 ± 0.76	5.20 ± 0.73	0.349
		p	0.432	0.700	

Data are means ± standard deviation. The difference between the parameters at baseline and after 6 months of treatment within each group, i.e., current smokers and non-smokers, were calculated using paired samples *t*-test. The differences between the current smokers and non-smokers were calculated using Student's *t*-test. Active treatment represents the combined groups of alirocumab and evolocumab.

Table 5
Functional and morphological properties of the arterial wall at baseline and after 6 months of active treatment of current smokers with diabetes (S + D) *versus* non-smokers without diabetes (NS + ND).

Parameter	Unit	Group (N)	Baseline	After 6 months active treatment	p
Flow-mediated dilation (Brachial artery)	%	S + D (16)	5.2 ± 6.0	7.5 ± 6.0	0.454
		NS + ND (53)	11.7 ± 6.2	13.9 ± 5.7	**0.0003**
		p	**0.008**	**0.003**	
Carotid intima-media thickness	mm	S + D (16)	0.73 ± 0.37	0.71 ± 0.23	0.137
		NS + ND (53)	0.65 ± 0.16	0.61 ± 0.28	0.027
		p	0.173	0.156	
Pulse wave velocity	m/s	S + D (16)	5.56 ± 0.61	5.32 ± 0.98	0.291
		NS + ND (53)	5.29 ± 0.69	5.14 ± 0.71	0.039
		p	0.341	0.437	

Data are mean ± standard deviation. The difference between the parameters at baseline and after 6 months of treatment within each group ((S + D) or (NS + ND)) were calculated using paired samples *t*-test. The differences between the current smokers with diabetes (S + D) and non-smokers without diabetes (NS + ND) were calculated using Student's *t*-test. Active treatment represents the combined groups of alirocumab and evolocumab.

morphological changes, where increased (i.e., improved) FMD was significantly negatively correlated with increased (i.e., worsened) c-IMT ($r = -0.419$; $p = 0.015$).

Discussion

In this study, we investigated the effects of alirocumab and evolocumab treatments in addition to standard treatments on the morphological and functional properties of the arterial wall in patients with premature and stable CAD and very high Lp(a) levels. Overall, there were no differences in the effects on lipids and lipoproteins between alirocumab and evolocumab. On the other hand, with FMD as an indicator of the functional properties of the arterial wall and c-IMT and PWV as indicators of the morphological properties of the arterial wall, these were only improved after treatment with evolocumab.

Both alirocumab and evolucomab significantly decreased total cholesterol and LDL-C by >60%, as well as significantly reducing triglycerides, Lp(a) and apoB. This was not unexpected, and is consistent with findings in previous studies [24,25,34].

As both alirocumab and evolocumab are fully human monoclonal antibodies that share the same pharmacodynamics and pharmacokinetic properties and mechanism of action, it is highly unlikely that these differences are a consequence of differences between the actions of these drugs. However, the mechanism by which PCSK9 inhibitors improve morphological and functional arterial wall properties is still not entirely understood [15]. In patients with increased CV risk, Maulucci et al. [15] showed that after 2 months of treatment with evolocumab, the improved endothelial function was proportional to the decreased LDL-C levels. The most likely mechanism of action here is inhibition of PCSK9-mediated LDL receptor degradation and recycling of LDL receptors back to the hepatic cell surface, which will lower serum LDL-C levels [15]. In a small clinical study, Leucker et al. [35] reported that in HIV patients with mean LDL-C values near optimal or above the goal and with other dyslipidaemias, PCSK9 inhibition with evolocumab significantly improved coronary endothelial function after 6 weeks of treatment [35].

To the best of our knowledge, there have not been any studies on the influence of alirocumab on endothelial function, either measured as FMD or using any other measures. Indeed, there are very limited data regarding the influence of PCSK9 inhibitors on the morphological properties of the arterial wall. A study by Hirai et al. [36] reported that in patients with hypercholesterolaemia and high CV risk, 12 months of evolocumab reduced the increase in c-IMT in the statin-taking patients. The main disadvantage of their study, however, was that it was retrospective, and they had no control group. They showed that the change in serum HDL-C and the baseline carotid mean c-IMT independently correlated with the change in mean c-IMT during treatment with evolocumab, whereas the changes in HDL-C and triglycerides were independently correlated with the change in maximum c-IMT. In patients with familial hypercholesterolaemia treated with PCSK9 inhibitors, Di Minno et al. [16] showed correlations between changes in small dense LDL particles (assessed according to the LDL score) and changes in oxidation markers, and improved endothelial function, in terms of improved carotid stiffness (by 21.4%) and carotid distensibility (by 62.8%). Lipid-lowering treatments and reduction of LDL-C by ameliorating oxidative stress and improving endothelial NO synthase imbalance are associated with improved endothelial function [16]. Further, endothelial dysfunction is commonly seen for patient with diabetes, and it can worsen with ischaemia and reperfusion injury. The mechanism here involves an imbalance between the constricting and relaxing factors derived from the

endothelium, such as NO and endothelin-1, which reduce arterial blood flow after reperfusion, and aggravate ischaemia [37].

In the present study, increased endothelial function correlated with increased apoA1 levels in the patients treated with evolocumab. Also, HDL-C increased significantly only in these evolocumab-treated patients, and improved FMD significantly correlated with decreased c-IMT. It has been shown that in macrophages evolocumab can increase the circulating levels of HDL-C and apoA1 and the expression of ATP-binding cassette transporter [22,38]. Changes in serum HDL-C levels have also been shown to negatively correlate with changes in c-IMT in patients taking statins [39].

Here, we also found greater improvements in the functional and morphological properties of the arterial wall in non-smokers. At the time of their inclusion in this study, these non-smokers already showed significantly better FMD compared to the smokers. It can also be hypothesised here that the cause of the poorer improvement in the functional and morphological arterial wall properties in the alirocumab-treated patients compared to patients treated with evolocumab was the greater proportions of smokers and diabetics in the alirocumab group. There are no data in the literature to date on the effects of PCSK9 inhibitors on endothelial function in patients who are either smokers or have diabetes, compared to non-smokers. Endothelial dysfunction due to smoking is triggered by reduced bioavailability of NO, and then by increased expression of adhesion molecules. Smoking-induced increased adherence of platelets and macrophages influences the development of a procoagulant and inflammatory environment. After *trans*-endothelial migration and activation, macrophages take up oxidised lipoproteins and differentiate into foam cells. Oxidised phospholipids trigger apoptosis of macrophages and lead to destabilisation and necrosis of atherosclerosis plaques. Smoking induces tissue remodelling and prothrombotic processes, together with activation of systemic inflammatory signals, which together lead to atherogenic vessel wall changes [40,41].

Regression and mostly stabilization of atherosclerotic plaques are morphological changes of the arterial wall that make plaques less prone to rupture, consequently leading to acute coronary syndrome. Serial intravascular ultrasonography imaging has demonstrated that in addition to statins, PCSK9 inhibitors can result in significantly greater atheroma regression than for statin monotherapies [42,43]. In the Global Assessment of Plaque Regression with a PCSK9 Antibody as Measured by Intravascular Ultrasound (GLAGOV) trial by Nicholls et al. [42], they reported that addition of evolocumab to statins in patients with CAD resulted in greater decrease in percent atheroma volume compared to placebo after 76 weeks of treatment. A retrospective optical coherence tomography study reported that treatment with evolocumab caused greater increases compared to statin monotherapy in fibrous cap thickening in patients after acute coronary syndrome [44]. The High-Resolution Assessment of Coronary Plaques in a Global Evolocumab Randomised Study (HUYGENS) by Nicholls et al. [45] showed that treatment with evolocumab 420 mg monthly significantly increased fibrous cap thickness and decreased lipids in comparison with statin monotherapy, as measured by optical coherence tomography [46].

A study by Gao et al. [47] and the Alirocumab for Thin-Cap Fibroatheroma in Patients with Coronary Artery Disease Estimated by Optical Coherence Tomography (ALTAIR) trial [48] reported that the addition of alirocumab to statins also has a role in promoting a more stable plaque phenotype, in addition to the LDL-C—lowering effects. Treatment with alirocumab 75 mg twice monthly in addition to atorvastatin or rosuvastatin was associated with greater reductions in LDL-C, greater increases in fibrous cap thickness, and greater decreases in maximum lipid arc compared with standard statin monotherapy [47]. On the other hand, in the ODYSSEY J-IVUS trial by Ako et al. [49] in Japanese patients with acute coronary syndrome and hypercholesterolaemia, they reported a trend to greater percent reduction in normalised total atheroma volume in the patients treated with alirocumab compared to standard therapy (i.e., atorvastatin or rosuvastatin), although as they indicated, this did not reach statistical significance.

There are also limitations to the current study. First, the study design was single blind randomized in contrast to more preferred double blind randomized trial. However, we would like to emphasize that this was the investigator initiated clinical study without any influence of pharmaceutical industry. Second, the sample size was relatively small, however adequately powered to detect the differences in changes of the arterial wall properties following treatment with PCSK9 inhibitors.

We showed here that the improvements in the morphological properties of the arterial wall significantly correlated with the decreases in total cholesterol, LDL-C and apoB. These serum lipids and apolipoproteins are important biomarkers for progression of atherosclerosis and are risk factors for CV events [50]. Hirai et al. [36] reported that the change in triglyceride levels positively correlated with the change in maximum c-IMT during treatment with evolocumab in patients who were already taking statins. They suggested that the effects of evolocumab on c-IMT can be partially explained by the reduction in triglycerides.

Conclusion

In contrast to the previous studies that have investigated the effect of PCSK9 inhibitors on FMD [15,16], the novelty of the current study is that it evaluated the effects of two PCSK9 inhibitors, alirocumab and evolocumab, on functional and morphological properties of the arterial wall in patients with stable CAD on maximal tolerated statins (with ezetimibe if needed) and high Lp(a) levels. The influence of alirocumab and evolocumab on the functional and morphological properties of the arterial wall appear not to be due only to their effects on lipoproteins. The results of the present study also indicate that other risk factors such as smoking, and diabetes can attenuate the beneficial effects of alirocumab and evolocumab on the functional properties of the arterial wall.

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Author contributions

Conception and design: ARL, MŠ. Analysis and interpretation of the data: ARL, MŠ. Drafting of the article: ARL, MŠ. Critical revision of the article for important intellectual content and final approval of the article: ARL, MŠ Provision of patients: ARL, MŠ. Obtaining of funding: MŠ. Collection and assembly of data: ARL, MŠ.

Clinical trial data

The data on the study are available at clinicaltrials.gov under registration number: NCT04613167. The full clinical trial protocol can be accessed on request from the corresponding author.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.athplu.2022.07.001.

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