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Proprotein convertase subtilisin/kexin type 9–CC-motif chemokine ligand 2 interactions link lipoprotein(a) composition to intermediate monocyte inflammation in coronary artery disease

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ABSTRACT

Background: Intermediate monocytes (IM) exhibit proinflammatory properties and contribute to atherosclerosis. Elevated lipoprotein(a) [Lp(a)] levels modulate monocyte behavior, while proprotein convertase subtilisin/kexin type 9 (PCSK9) has been implicated in inflammatory pathways beyond lipid metabolism. The effects of PCSK9 inhibition on monocyte subset distribution in high-risk coronary artery disease patients remain unclear.
Objective: To assess the effects of lipoprotein fractions and PCSK9 inhibitor (PCSK9i) therapy on monocyte subset distribution in patients with stable coronary artery disease and highly elevated Lp(a) levels.
Methods: We followed 100 statin-treated patients in the stable phase after myocardial infarction with highly elevated Lp(a), randomized to PCSK9i or placebo for six months. Biochemical, genetic, and cellular analyses were performed at baseline and follow-up.
Results: At baseline, IM levels correlated with total cholesterol ($\rho = -0.202$, $p = 0.044$), triglycerides ($\rho = -0.324$, $p < 0.001$), apolipoprotein A1 ($\rho = 0.241$, $p = 0.016$), and PCSK9 concentrations ($\rho = -0.282$, $p = 0.006$). PCSK9 levels were positively associated with CC-motif chemokine ligand 2 (CCL2) ($\rho = 0.298$, $p = 0.005$), a marker of monocyte recruitment. PCSK9i therapy did not alter monocyte subset distribution. After treatment, only the association between IM and Lp(a) remained significant ($\rho = -0.258$, $p = 0.041$). KIV-2 repeat number inversely correlated with CCL2 levels ($\rho = -0.319$, $p = 0.011$).
Conclusion: In high-risk patients, PCSK9 inhibition modulates monocyte–lipoprotein interactions without affecting the monocyte subset distribution. PCSK9 may promote vascular inflammation through CCL2 regulation, which appears more closely related to Lp(a) composition than its circulating concentration.
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1. Introduction

The development of atherosclerosis from endothelial dysfunction to acute complications is associated with low-grade inflammation [1]. Monocytes are one of the most important cell types of the immune system involved in the process of atherosclerosis. The link between monocytes and atherosclerosis is unequivocal, and an increased number of monocytes is causally related to the complications of atherosclerosis [2]. Monocytes represent a very heterogeneous population of cells that play different roles in the process of atherosclerotic lesion development. Monocytes can be divided into $CD14^{++}CD16^{-}$ (classical monocytes (CM)), $CD14^{++}CD16^{+}$ (intermediate monocytes (IM)) and $CD14^{+}CD16^{++}$ (non-classical monocytes (NCM)) [3].

Despite the fact that the IM subpopulation is the smallest among monocytes, their concentration has been shown to be an independent predictive factor for future coronary events in patients referred for elective coronary angiography [4]. The proatherogenic potential of IM is most likely related to their ability to increase synthesis of tumor necrosis factor-alpha (TNF-α) compared to other monocytes [5].

Activation of the endothelium allows infiltration of monocytes into the vessel wall, where they are captured and gradually transformed into macrophages [6]. CC-motif chemokine ligand 2 (CCL2) is one of the strongest recruitment signals for monocytes to the site of inflammation [7]. CCL2 is produced by endothelial cells, smooth muscle cells, and macrophages in atherosclerotic lesions and binds to C–C chemokine receptor type 2 (CCR2) on circulating monocytes [7]. CCR2 enables the passage of monocytes into the subendothelial space [8]. Moreover, CCR2 expression is increased in patients with hypercholesterolemia [9].

In addition to its proatherogenic and prothrombogenic properties, lipoprotein(a) (Lp(a)) has well-described proinflammatory effects [10]. It is also an independent risk factor for cardiovascular disease regardless of LDL cholesterol (LDL-C) levels, and has been shown to affect the distribution of the monocyte subsets. Patients with stable coronary artery disease treated with statins and with Lp(a) values above 500 mg/L had significantly more IM than those with values below 500 mg/L. However, there were no differences in the proportion of CM and NCM between these two groups [11].

There is very little data on the CCR2 expression in patients with elevated Lp(a) levels. In a very small study, there were no differences between patients with Lp(a) above 500 mg/L and LDL-C levels above 4.5 mmol/L with no lipid-lowering therapy compared to healthy subjects [12]. In the same study, the expression of CCR2 was shown to be dependent on LDL-C, male gender, and systolic blood pressure in patients with various cardiovascular risk factors.

Even a short-term interruption of statin treatment in young patients after myocardial infarction caused an increase in the concentration of IM, which suggests that statins also possess an anti-inflammatory effect [13]. In patients with untreated familial hypercholesterolemia (FH), the monocyte distribution with respect to CD14 and CD16 expression was comparable to healthy subjects. However, the CCR2 expression on CM was increased in FH patients and associated with LDL-C levels [14]. Treatment with proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors (PCSK9i) did not alter the distribution of the monocyte subsets, but there was a significant downregulation of CCR2 expression on the surface of monocytes [12].

In the current study, our aim was first, to determine how individual lipoproteins affect the distribution of the monocyte subsets, and second to identify whether treatment with PCSK9i, which decreases both LDL-C and Lp(a), affects the monocyte subsets in terms of CD14 and CD16 distribution and CCL2 expression in high-risk patients.

2. Patients and methods

2.1. Patients

The current study was performed using a patient cohort as described previously [15]. Briefly, 100 patients in the stable phase after myocardial infarction (first acute coronary syndrome before the age of 55) and elevated Lp(a) (levels above 1000 mg/L or a Lp(a) level above 600 mg/L and a LDL-C level of more than 2.6 mmol/L) 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 eight weeks before inclusion in the study. Patients were randomised (according to a randomisation list) to three groups: standard lipid-lowering therapy with no PCSK9i (placebo; N = 31), or alirocumab 150 mg SC (N = 35) or evolocumab 140 mg SC (N = 34), every two weeks. Approval for this study was obtained from the National Medical Ethics Committee of the Republic of Slovenia (reference number: KME 0120-357/2018/8). This study is registered with ClinicalTrials under the number NCT04613167. All patients signed a written informed consent prior to inclusion in the study.

2.2. Biochemical analysis

Blood for laboratory analysis was drawn in the morning after 12 h of fasting. Samples were collected from the antecubital vein into 5 mL vacuum-sealed tubes containing clot activator (Vacutube; LT Burnik, Skaručna, Slovenia). Blood samples were centrifuged the at 2000 g for 15 min to separate the serum. In fresh serum samples, 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 analyser (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 is minimal. The Friedewald formula [16] was used to calculate LDL-C.

Total PCSK9 was measured in serum samples using a sandwich enzyme-linked immunosorbent assay (ELISA) (Human Proprotein Convertase 9/PCSK9 Quantikine® ELISA Kit, R&D Systems, Minneapolis, MN, USA) on a Sunrise microplate reader and Magellan software (Tecan, Männedorf, Switzerland) as instructed by the manufacturer. Sensitivity and assay range provided by the manufacturer were 0.219 ng/mL and 0.6–40 ng/mL, respectively. Samples were diluted 1:20 with calibrator diluent. Samples exceeding the upper limit were diluted 1:40 and reanalysed. The assay used measured total PCSK9 in serum, so it was not possible to distinguish between bound and unbound PCSK9.

The LEGENDplex™ Human Vascular Inflammation Panel 2 (BioLegend, San Diego, California) was used to measure CCL2 concentration in plasma samples. The concentration of each analyte was determined using standard curves. The tests were performed on a filter plate according to the manufacturer's protocol. All samples were measured in duplicate using a BD FACSLyric™ System flow cytometer (Becton, Dickinson and Company, New Jersey, USA). At least 5000 beads were analysed for each sample. The FCS files of the assays were analysed with the data analysis software LEGENDplex™ from BioLegend.

2.3. Genetic analysis

Genomic DNA was extracted from venous blood samples using FlexiGene DNA kit 250 (Qiagen, Hilden, Germany), following the manufacturer instructions. The analysis of *LPA* kringle IV type 2 (KIV-2) repeats was performed with TaqMan Universal PCR Master Mix and the QuantStudio 7 Flex real-time PCR system (all Applied Biosystems, Foster City, CA, USA). The KIV-2 repeats were analysed in triplicate using multiplex qPCR with a custom TaqMan expression assay for exon 5 of the *LPA* gene, and TaqMan Copy Number Reference Assay for the RNAseP gene that was used as a single copy reference gene (both Applied Biosystems, Foster City, CA, USA). If there were any discrepancies of >0.25 in the Ct values in replicates for either assay in

individual samples, the sample was reanalysed. The relative number of KIV-2 repeats representing average value of repeats on both alleles in the individual patients was determined using the CopyCaller v2.1 software (Applied Biosystems, Foster, CA, USA).

2.4. Flow cytometry

Flow cytometry was performed on cryopreserved peripheral blood mononuclear cells (PBMCs), which were isolated from EDTA blood samples drawn as described under Biochemical analysis section following the previously described protocol [17]. Cells were isolated by density gradient centrifugation using Lymphocyte Separation Medium 1077 (Promocell, Heidelberg, Germany) and SepMate-15 Tubes (Stemcell, Vancouver, Canada). After washing, the PBMCs were resuspended in 90% FBS Gold (Seraglob, Schaffhausen, Switzerland) supplemented with 10% dimethyl sulfoxide (DMSO, >99.9% purity, Sigma–Aldrich, St. Louis, USA) and frozen at −80 °C in a CoolCell reduced-scale freezing container (Corning, Corning, USA). Afterwards, the cryo-tubes were transferred into liquid nitrogen tanks and stored there until thawing for flow cytometry analysis.

On the day of analysis, cryopreserved cells were rapidly thawed in 25 mL of cold PBS (Sigma-Aldrich, St. Louis, USA) and centrifuged (500×*g*, 5 min). The supernatant was discarded, and the pellet was resuspended in 1 mL PBS. A 70 μL aliquot was taken for cell counting using a Sysmex haematology analyzer (protocol: whole blood differential count), and both total cell numbers and relative population distributions were documented. After another wash step, cells were resuspended in PBS at a concentration of 2×10^7 cells/mL.

For staining, 5 μL of the FACS staining mix and 5 μL of the prepared cell suspension were added to each well of a 96-well U-bottom-plate. Samples were processed in duplicates. Monocyte subsets were identified using antibodies targeting CD14 (PerCP), CD16 (APC/Fire750), CD45 (BV605), all from BioLegend (except CD34 from BD Biosciences), with 0.2 μL of each antibody used per sample. Following a 15-min incubation at room temperature in the dark, 220 μL of 1% paraformaldehyde (PFA) in PBS was added to each well to fix the cells. Samples were analysed on the same day using a Attune 3-laser flow cytometry system (ThermoFisher, Waltham, USA). All cytometric data were acquired and saved immediately following analysis. Analysis was performed using Attune Cytometric Software v.5.3.1.

2.5. Statistical analysis

The normality of the distribution of continuous variables was analysed with the Shapiro–Wilk test. The median with interquartile range or the mean with standard deviation was used to describe the continuous variables. The 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 the 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 the changes following the placebo and PCSK9i treatment. The correlations between the continuous variables were calculated using the Spearman's ρ coefficient. All statistical tests were two-sided. G Power was used to perform the power calculations. We used 0.05 as the level of statistical significance. IBM SPSS Statistics version 27.0 (IBM Corporation, New York, NY, USA) was used to perform the statistical analyses. Graphs were generated using GraphPad Prism 8 (San Diego, CA, USA).

3. Results

3.1. Patients' characteristics

The study included 100 patients with clinically stable coronary artery disease at least six months post myocardial infarction, and with their first coronary event occurring before the age of 55 years. The patients were randomised into three groups; placebo group (N = 31), alirocumab group (N = 35) and evolocumab group (N = 34). Since no differences were observed between the groups receiving the active substance either at the beginning of the study or after treatment, we combined these two groups to increase the statistical power. Baseline patient characteristics are shown in Table 1. The clinical parameters, lipid indicators, and inflammatory parameters were comparable across all groups.

3.2. Effect of PCSK9i on lipoprotein fractions, C-reactive protein and leukocyte monocyte subset distribution

In the placebo group, the six months of treatment had no significant effect on lipoprotein fractions, except for HDL cholesterol, which increased significantly ($p = 0.010$). In contrast, treatment with PCSK9i led to significant changes across all lipoprotein fractions ($p < 0.0001$ for each). Notably, hs-CRP concentrations – already low at baseline (<1 mg/L) – remained unchanged in all groups, as did leukocyte and monocyte counts (Table 2).

3.3. The association of lipoproteins and the monocyte subtypes before and after treatment with PCSK9i

Monocyte subsets were analysed by flow cytometry gated for CD45, CD14 and CD16 (Fig. 1). Prior to PCSK9i treatment, significant correlations were observed between the lipoprotein levels and the monocyte subtypes. Both, TC and TG levels showed negative correlations with IM ($\rho = -0.202$, $p = 0.044$ and $\rho = -0.324$, $p < 0.001$, respectively), while apoA1 positively correlated with IM levels ($\rho = 0.241$, $p = 0.016$). Additionally, HDL-C levels were positively correlated with the CCL2 concentrations ($\rho = 0.211$, $p = 0.045$). No other associations were observed between the lipoprotein concentrations and the monocyte subtypes (Table 3).

After treatment with PCSK9i, a significant negative correlation between Lp(a) and IM levels ($\rho = -0.258$, $p = 0.041$) was observed. Moreover, negative correlation between KIV-2 repeats and CCL2 levels ($\rho = -0.319$, $p = 0.011$) was found. No such correlations were found in placebo group (Fig. 2).

Table 1
The baseline patients' clinical and biochemical characteristics.

	Placebo group N = 31	Active treatment group N = 69	*p*-value
Age (years)	47.6 ± 9.5	50.7 ± 7.2	0.350
Male gender, n (%)	26 (84)	59 (87)	0.753
Diabetes mellitus, n (%)	7 (20)	7 (10)	0.190
Current smokers, n (%)	3 (9.7)	9 (13)	0.547
BMI (kg/m^2)	28.4 ± 3.5	28.6 ± 3.8	0.863
Leukocytes (10^9/L)	7.1 ± 1.9	7.1 ± 1.7	0.935
Monocytes (10^9/L)	0.45 ± 0.07	0.47 ± 0.08	0.921
Blood glucose (mmol/L)	6.7 ± 3.4	6.2 ± 1.7	0.375
TC (mmol/L)	4.3 ± 1.0	4.2 ± 0.8	0.812
LDL-C (mmol/L)	2.4 ± 0.9	2.3 ± 0.7	0.871
HDL-C (mmol/L)	1.1 ± 0.2	1.2 ± 0.3	0.227
TG (mmol/L)	1.6 (1.0-2.0)	1.7 (1.0-2.1)	0.840
Lp(a) (mg/L)	1491 (1185-1739)	1416 (1201-1781)	0.915
KIV-2 repeats	9.5 (8.5-10.5)	10.2 (8.6-11.0)	0.270
hsCRP (mg/L)	0.8 (0.4-2.2)	0.9 (0.4-1.9)	0.592
PCSK9 (ng/L)	240.3 (195.1-364.4)	285.7 (210.0-401.3)	0.265

BMI, body mass index; HDL-C, high density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein(a); PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; TG, triglycerides.

Table 2
The biochemical and inflammatory parameters at baseline and after 6 months of treatment.

	Group	Baseline	After 6 months	*p*-value
Blood glucose (mmol/L)	Placebo	6.7 ± 3.4	6.2 ± 2.2	0.324
	Active treatment	6.2 ± 1.7	6.2 ± 1.7	0.764
TC (mmol/L)	Placebo	4.3 ± 1.0	4.4 ± 0.9	**0.425**
	Active treatment	4.2 ± 0.8	2.6 ± 1.0	****<0.001**
LDL-C (mmol/L)	Placebo	2.4 ± 0.9	2.4 ± 0.8	**0.735**
	Active treatment	2.3 ± 0.7	0.9 ± 0.8	****<0.001**
HDL-C (mmol/L)	Placebo	1.1 ± 0.2	1.2 ± 0.3	**0.010**
	Active treatment	1.2 ± 0.2	1.3 ± 0.3	****<0.001**
TG (mmol/L)	Placebo	1.6 (1.0-2.2)	1.6 (1.1-2.1)	**0.600**
	Active treatment	1.5 (1.1-2.1)	1.2 (0.8-1.8)	****<0.001**
Lp(a) (mg/L)	Placebo	1491 (1185-1739)	1397 (1224-1574)	**0.701**
	Active treatment	1416 (1201-1781)	1133 (820-1664)	****<0.001**
hsCRP (mg/L)	Placebo	0.8 (0.4-2.2)	0.8 (0.4-2.0)	0.542
	Active treatment	0.9 (0.4-1.9)	0.8 (0.3-1.7)	0.666
Leukocytes (10^9/L)	Placebo	7.1 ± 1.9	7.1 ± 1.8	0.975
	Active treatment	7.1 ± 1.7	7.2 ± 1.8	0.865
Monocytes (10^9/L)	Placebo	0.45 ± 0.07	0.46 ± 0.07	0.916
	Active treatment	0.47 ± 0.08	0.46 ± 0.09	0.863

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. 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. Values $^{**}p < 0.001$ are statistically significant.
HDL-C, high density lipoprotein cholesterol; hsCRP, high-sensitivity C-reactive protein; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein(a); TC, total cholesterol; TG, triglycerides.

3.4. Effect of PCSK9i on the monocyte subsets' distribution

Both, placebo and PCSK9i treatment significantly increased the CCL2 expression ($p < 0.001$ for both), while no other significant changes were observed (Table 4).

3.5. Alterations in the monocyte subpopulations in relation to changes in lipoproteins

The relative changes in IM levels showed negative correlations with changes in TC ($\rho = -0.315$, $p = 0.011$), LDL-C ($\rho = -0.279$, $p = 0.026$), and apoB ($\rho = -0.400$, $p = 0.001$) levels. No other significant associations were observed between the lipoprotein changes and the alterations of other monocyte subpopulations (Fig. 3).

Monocyte subpopulation distributions showed no association with Lp(a) levels below and above the median value, either at baseline or at the end of the study (results not shown).

Regarding the median KIV-2 repeats, patients with higher number of KIV-2 repeats had significantly elevated NCM levels before treatment ($p = 0.046$), along with a trend toward statistically significant higher IM levels ($p = 0.087$). However, these differences were no longer observed after treatment with PCSK9i (Fig. 4).

A significant negative correlation between Lp(a) and number of KIV-2 repeats was observed before ($\rho = -0.601$, $p < 0.001$) and after ($\rho = -0.424$, $p < 0.001$) treatment with PCSK9i.

As for the CCL2 expression regarding median KIV-2 repeats, baseline values did not differ between the groups. However, after PCSK9i treatment, the patients with fewer KIV-2 repeats possess higher CCL2 expression (Fig. 5).

4. Discussion

Our study showed that in high-risk patients with coronary artery disease, the addition of PCSK9i to maximally tolerated statin therapy did not significantly alter the distribution of monocyte subtypes (CM, IM, NCM). Prior to treatment, IM levels correlated with several lipid parameters, including TC, TG, apoA1, and PCSK9. Furthermore, PCSK9 levels were associated with CCL2 expression, a chemokine critical for monocyte recruitment. After PCSK9i treatment, these associations were largely attenuated, with only the correlation between IM and Lp(a) levels remaining significant. Although monocyte subtype distribution was independent of Lp(a) concentration, it strongly correlated with the number of KIV-2 repeats, a marker of Lp(a) atherogenicity that may be superior to Lp(a) concentration alone [10,18]. Longitudinal analysis showed that changes in IM levels correlated with changes in TC, LDL-C, and apoB concentrations, whereas other associations remained non-significant.

The effects of PCSK9i or placebo on lipoproteins and hs-CRP in our cohort were previously reported [19], with results consistent with larger studies of alirocumab and evolocumab [20,21]. While Krychtiuk et al. [11] reported Lp(a)-dependent changes in monocyte subtype concentrations, particularly elevated IM levels, we observed no association between monocyte subtypes and median Lp(a) levels either at baseline or after PCSK9i treatment. This discrepancy may reflect fundamental cohort differences, as our patients had more than twofold higher baseline Lp(a) levels than those in the study by Krychtiuk et al. Moreover, despite an approximately 25% reduction with PCSK9i, post-treatment Lp(a) levels in our cohort remained substantially higher than in their study. In the same patients, monocyte subtype distribution showed no correlation with TC, LDL-C, HDL-C, or TG concentrations. However, concentrations of small HDL-C [22] and small LDL-C [23] particles were associated with levels of the proinflammatory NCM independently of other laboratory and clinical parameters.

The stability of hs-CRP levels in our cohort, which remained below 1 mg/L throughout the study, likely reflects the anti-inflammatory effects of concomitant high-dose statin therapy combined with angiotensin-converting enzyme inhibitors and acetylsalicylic acid in all participants. These findings are consistent with those of Bernelot Moens et al. [14], who also reported no changes in monocyte subtype distribution in statin-naïve patients with familial hypercholesterolemia (FH), despite substantially higher baseline TC and LDL-C levels compared with our cohort. Notably, both studies reported baseline hs-CRP values below 1 mg/L that remained unchanged after PCSK9i treatment. In contrast, a significant reduction in hs-CRP was observed in statin-naïve patients not receiving other cardiovascular preventive medications who were treated with PCSK9i [24]. Importantly, baseline hs-CRP levels in that study were nearly threefold higher than those observed in our cohort [24].

Our finding that PCSK9i did not alter monocyte subtype distribution is consistent with the study by Bernelot Moens et al. [14] in statin-naïve patients with FH, a cohort with significantly higher TC and LDL-C levels than ours. However, their study demonstrated a reduction in monocyte transendothelial migration (TEM) capacity, likely due to decreased expression of CCR2 on the monocyte surface. After PCSK9i therapy, monocyte TEM capacity in statin-naïve patients decreased to levels comparable to those observed in FH patients treated with statins alone, correlating with the degree of LDL-C reduction. Notably, achieved LDL-C levels in both groups in the Bernelot Moens study were comparable to baseline LDL-C levels in our intensively pretreated cohort. Although these findings might suggest that combined statin and PCSK9i therapy could further reduce CCR2 expression and TEM capacity, such interpretation should be made cautiously. Despite PCSK9i treatment, patients in our study still had Lp(a) levels within a highly atherogenic range.

In our study, the concentration of IM correlated consistently with the

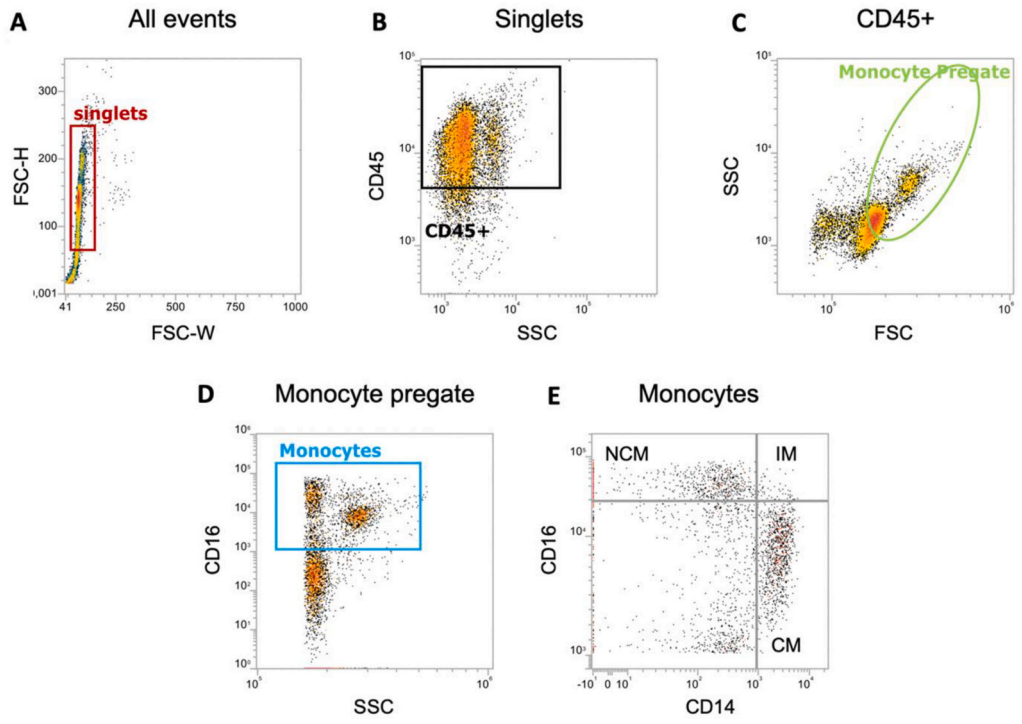

Fig. 1. Gating strategy used for monocyte subset discrimination. Initial gating on forward scatter-height (FSC-H) and weight (FSC-W) was used to exclude doublets and select single cells (**A**). From these, $CD45^{+}$ leukocytes were identified (**B**). The monocyte population was then pregated from the $CD45^{+}$ cells using their typical sideward scatter (SSC) and forward (FSC) profile (**C**) and subsequent gate (**D**) was used to exclude possible contamination and confirm the monocyte population. Final subset discrimination was achieved based on their CD14 and CD16 surface expression, defining classical monocytes ($CD14^{++}CD16^{-}$; CM), intermediate monocytes ($CD14^{++}CD16^{++}$; IM) and non-classical monocytes ($CD14^{+}CD16^{++}$; NCM) (**E**).

Table 3
The correlations between the lipoproteins and the monocyte subtypes before PCSK9i treatment.

	TC (mmol/L)	LDL-C (mmol/L)	HDL-C (mmol/L)	TG (mmol/L)	Lp(a) (mg/L)	KIV-2 repeats	PCSK9 (ng/L)	ApoA1 (g/L)	ApoB (g/L)
NCM (%)	−0.017 (0.863)	−0.002	0.027 (0.792)	0.018 (0.859)	−0.120	0.129	0.195	0.007	−0.002
		0.988			0.238	0.202	0.059	0.942	0.988
IM (%)	**−0.202**	−0.067	0.046 (0.650)	**−0.324**	−0.107	−0.081	**−0.282**	**0.241**	−0.171
	***0.044**	0.508		****<0.001**	0.290	0.429	***0.006**	***0.016**	0.093
CM (%)	−0.081	0.003	−0.087	−0.080	0.015	−0.069	0.141	0.156	−0.066
	0.423	0.975	0.389	0.428	0.882	0.497	0.172	0.122	0.515
CCL2 (pg/mL)	0.055	−0.025	**0.211**	−0.023	0.097	0.173	**0.298**	0.172	−0.178
	0.602	0.811	***0.045**	0.827	0.364	0.105	***0.005**	0.103	0.096

Values are rho coefficient, (*p*-value), $*p < 0.05$, $**p < 0.001$. Spearman's correlation analysis was used to determine the statistically significant correlations.
ApoA1, apolipoprotein A1; ApoB, apolipoprotein B; CCL2, CC-motif chemokine ligand 2; CM, classical monocyte; HDL-C, high density lipoprotein cholesterol; IM, intermediate monocyte; LDL-C, low density lipoprotein cholesterol; Lp(a), lipoprotein(a); NCM, non-classical monocyte; PCSK9, proprotein convertase subtilisin/kexin type 9; TC, total cholesterol; TG, triglycerides.

lipoprotein parameters, showing negative association with TC and TG alongside positive correlations with apoA1 at baseline. The negative association between IM on the one hand and TC and TG on the other is counterintuitive, as a positive correlation would be expected. We acknowledge that this finding is surprising and may reflect the unique characteristics of our cohort, which featured uniformly extremely high Lp(a) levels. Such marked elevations in Lp(a) could obscure typical Lp (a)-monocyte subtype relationship while revealing associations between IM and other lipid parameters that might otherwise remain undetected in populations with a broader range of Lp(a) concentrations. A similar association between IM and LDL-C levels was reported in a study of 67 patients with clinically evident atherosclerotic disease [25]. However, unlike our cohort, where all patients received statins and secondary preventions medications prior to PCSK9i therapy, their study population were not treated with statins or other drugs in secondary prevention. Importantly, their study did not report Lp(a) concentrations, limiting direct comparison of the cohorts. However, these are all factors that significantly influence the association between monocytes or monocyte subtypes and lipoproteins. Therefore, our results cannot be generalized to other populations and should be interpreted within the context of this specific patient group with coronary artery disease and markedly elevated Lp(a).

The relationship between IM and Lp(a) demonstrated complex dynamics. While Krychtiuk et al. [11] reported concentration-dependent associations between Lp(a) and monocyte subsets in patients with stable atherosclerosis, we did not observe such associations at baseline in our intensively lipid-lowered cohort. This discrepancy likely reflects differences in cohort characteristics, most notably the more than twofold higher baseline Lp(a) levels in our patients, which remained markedly elevated despite approximately 25% reduction with PCSK9i. However, after PCSK9i treatment an association emerged between Lp(a) and IM levels, particularly at achieved LDL-C concentrations of approximately 1 mmol/L. This observation suggests that Lp(a) may become more pathogenically relevant when other lipoprotein risk

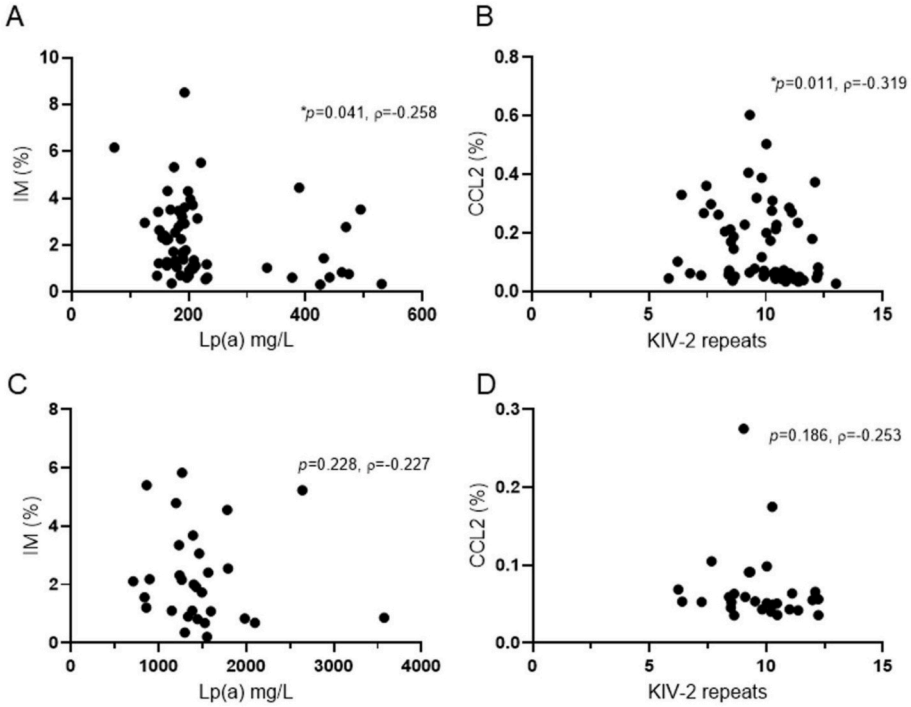

Fig. 2. The significant correlations between the intermediate monocytes (IM) and lipoprotein(a) (Lp(a)) levels (**A** and **C**), and the number of KIV-2 repeats and CC-motif chemokine ligand 2 (CCL2) levels (**B** and **D**) in patients treated with PCSK9i (**A** and **B**) and in patients receiving placebo (**C** and **D**). Spearman's correlation analysis was used (ρ, rho coefficient, *p < 0.05).

Table 4
The effect of PCSK9i and placebo on the distribution of the monocyte subsets.

	Group	Baseline	After 6 months	*p-value*
NCM (%)	Placebo	1.2090 (0.5740-2.4940)	1.1870 (0.4715-1.8860)	0.652
	Active treatment	0.8980 (0.4193-1.7010)	1.0025 (0.5605-1.8441)	0.820
	p-value	0.220	0.899	0.584
IM (%)	Placebo	2.0705 (1.1480-3.2445)	2.1721 (0.8590-3.0615)	0.544
	Active treatment	2.0030 (1.0905-2.8343)	1.7443 (1.0018-3.3053)	0.963
	p-value	0.923	0.784	0.763
CM (%)	Placebo	10.3220 (7.1950-15.5375)	11.6060 (9.0780-14.9615)	0.281
	Active treatment	11.3610 (9.2553-14.4978)	10.6348 (8.1315-14.3635)	0.139
	p-value	0.369	0.280	0.066
CCL2 (pg/mL)	Placebo	**0.023 (0.009–0.048)**	**0.053 (0.044–0.067)**	****<0.001**
	Active treatment	**0.028 (0.023–0.057)**	**0.073 (0.054–0.236)**	****<0.001**
	p-value	**0.003**	**0.001**	0.740

Data are medians (lower-upper quartile) or means ± standard deviation. The differences between the two groups were calculated with *t*-test or Mann-Whitney *U* test. 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. Values **$p < 0.001$ are statistically significant.
CCL2, CC-motif chemokine ligand 2; CM, classical monocyte; IM, intermediate monocyte; NCM, non-classical monocyte.

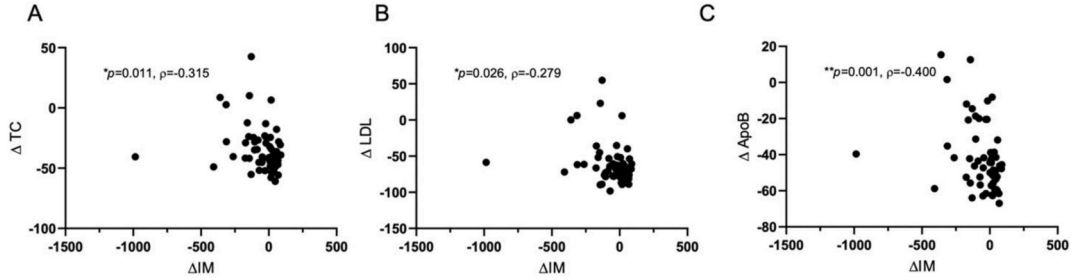

Fig. 3. The significant correlations between the changes in the intermediate monocytes (IM) levels and changes in total cholesterol (TC) (**A**), low-density lipoprotein cholesterol (LDL-C) (**B**), and apolipoprotein B (apoB) (**C**) concentrations were observed. Spearman's correlation analysis was used. Δ, change between baseline and after PCSK9i treatment calculated as (value after PCSK9i-value at baseline)/value at baseline and expressed in % (ρ, rho coefficient; *$p < 0.05$, **$p < 0.001$).

factors are well controlled.

Most notably, we observed a significant negative association

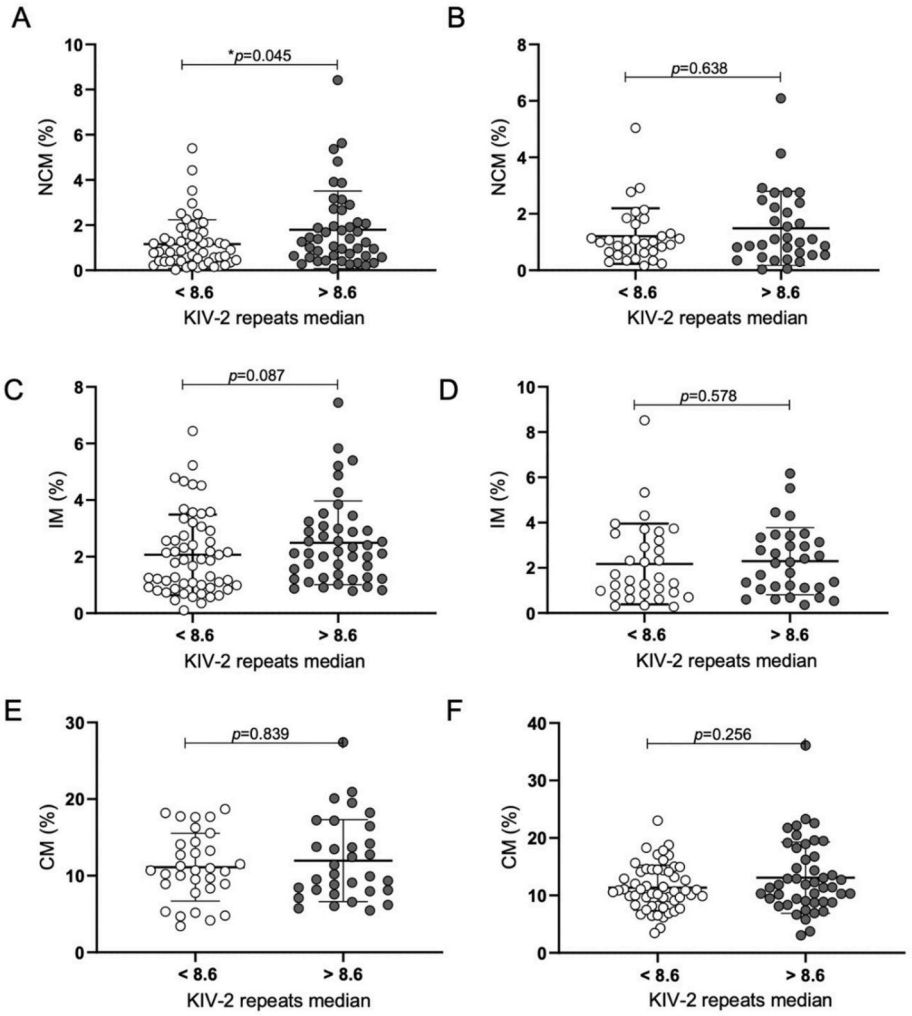

Fig. 4. The difference in monocyte subtype levels between the groups of patients divided by the median of KIV-2 repeats. The levels of non-classical monocytes (NMC) before **(A)** and after **(B)** treatment with PCSK9i, intermediate monocytes (IM) before **(C)** and after **(D)** treatment with PCSK9i and classical monocytes (CM) before (**E**) and after (**F**) treatment with PCSK9i. Value $*p < 0.05$ is statistically significant.

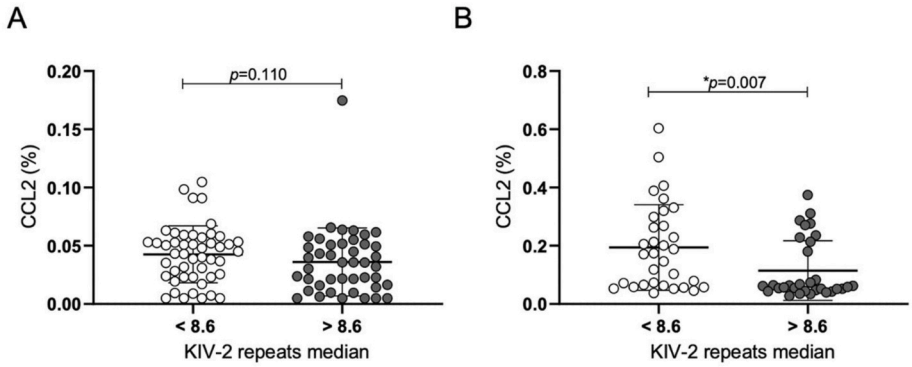

Fig. 5. The expression of CCL between the groups of patients divided by the median of KIV-2 repeats before (**A**) and after (**B**) treatment with PCSK9i. Value $*p < 0.05$ is statistically significant.

between the number of KIV-2 repeats and IM levels following PCSK9i treatment. These findings have important clinical implications given the established inverse relationship between KIV-2 repeat number and Lp(a) atherogenicity. As demonstrated previously [26], Lp(a) isoforms with fewer KIV-2 repeats are associated with an increased risk of thrombosis following plaque rupture. Our findings therefore suggest that redistribution toward proinflammatory monocyte subtypes may be driven primarily by qualitative lipoprotein characteristics rather than by absolute lipoprotein concentrations. Consistent with this interpretation, monocyte distribution in our cohort correlated more strongly with the number of KIV-2 repeats than with circulating Lp(a) concentrations despite uniformly elevated Lp(a) levels. This observation highlights the importance of Lp(a) particle composition in influencing monocyte behavior.

The nature of the relationship between Lp(a) and monocyte subtype distribution remains uncertain, particularly whether this relationship follows a linear pattern or plateaus beyond a certain Lp(a) threshold. Addressing this question would require studies including patients across a very wide range of Lp(a) concentrations, from extremely low to the very high levels observed in our cohort. More definitive insights may emerge from trials of novel therapies capable of reducing Lp(a) concentrations by more than 90% [27]. Importantly, such trials already include patients similar to those in our study—individuals receiving combined statin and PCSK9i therapy with very low LDL-C levels.

The observed correlation between IM levels and circulating PCSK9 concentrations represents another noteworthy finding and contrasts with the CM and NCM associations reported by Krychtiuk et al. [28] in statin-treated patients. The clinical relevance of this observation is supported by evidence demonstrating that IM concentration is the only monocyte subtype independently predictive of future cardiovascular events [4]. In addition to its central role in LDL-C metabolism, circulating PCSK9 concentration has also been identified as an independent predictor of future cardiovascular events after adjustment for TC, LDL-C, HDL-C, TG, and statin therapy [29]. This relationship is notable given the dual effect of statins in upregulating both LDL receptors and PCSK9 expression [30], with increases in PCSK9 inversely proportional to LDL-C reduction [31]. Although statins influence both monocyte distribution and PCSK9 levels, the pre-treatment correlations observed in our study suggest that monocyte patterns may retain prognostic significance even in optimally statin-treated patients.

The post-treatment association between IM and Lp(a) concentration suggests that further reduction of Lp(a) might promote a more anti-atherogenic monocyte profile. This hypothesis is supported by the potential interaction between PCSK9 and the CCL2/CCR2 axis, one of the key proinflammatory pathways in atherosclerosis [32]. The pre-treatment correlation between PCSK9 and CCL2 observed in our study suggests that PCSK9 may promote atherosclerosis through multiple mechanisms: by increasing CCL2 levels, a validated cardiovascular risk predictor [33], and by upregulating its receptor CCR2 [34], thereby enhancing monocyte recruitment and proinflammatory transformation. The additional association between CCL2 and the number of KIV-2 repeats further highlights an important dimension of Lp(a) pathogenicity: its proinflammatory effects appear to depend not only on concentration but also on intrinsic particle characteristics. This dual influence may explain why monocyte distribution in our cohort correlated more strongly with the number of KIV-2 repeats than with absolute Lp(a) concentrations.

We cannot fully explain the significant increase in CCL2 concentration observed in both the PCSK9i and placebo groups, with no differences between them. Several possibilities warrant consideration. First, our analysis measured the CCL2 concentration but not its activity, which may not directly reflect its proinflammatory potential. Second, although seasonal variation is a theoretical possibility, this is unlikely because patient randomization accounted for such variables. Third, and most importantly, only Lp(a) concentrations changed significantly during the study, whereas the number of KIV2 repeats, showing a stronger association with CCL2 expression, remained unchanged.

Several limitations should be considered when interpreting these findings. The relatively small sample size reflects the stringent inclusion criteria for Lp(a) levels, although this also resulted in a highly homogeneous study population. Monocyte subset analysis was performed on cryopreserved PBMCs, which may affect viability and recovery of certain monocyte subsets, particularly $CD16^{+}$ populations (IM and NCM), potentially influencing the reported distribution [35]. This effect may be more pronounced in patients with hypercholesterolemia, whose monocytes may be more susceptible to cryopreservation-induced apoptosis compared with those from individuals with lower LDL-C levels [36]. Finally, the inability to measure free PCSK9 concentrations after treatment due to limited specificity of the ELISA assay represents an additional technical limitation. Confirmation and broader interpretation of our findings will require multicenter studies with ethnically diverse cohorts and longitudinal clinical outcome data.

5. Conclusions

To the best of our knowledge, this is the first study examining the monocyte subtypes' associations with the lipoproteins in patients with stable coronary artery disease, along with the effects of PCSK9i on these relationships. Our cohort is particularly distinctive, comprising patients who have had an acute coronary event before age 55, received guideline-directed therapy [37], maintained relatively controlled risk factors, yet exhibited markedly elevated Lp(a) values. Several key observations emerged from our investigation. First, while PCSK9i showed no effect on overall monocyte distribution, we identified a significant correlation between IM and circulating PCSK9 levels. Second, our data suggest PCSK9 may exert proinflammatory effects through regulation of the CCL2, one of the most important chemokines in the atherosclerotic inflammation. Most intriguingly, the CCL2 expression patterns were more closely tied to Lp(a) quality, as determined by the number of KIV-2 repeats, than to absolute Lp(a) concentration.

Since our patients maintained substantial Lp(a) elevations despite treatment with PCSK9i, further research is needed to determine how treatment with drugs that specifically reduce Lp(a) affects the distribution of monocyte subtypes and their properties.

Ethical approval

The study protocol was approved by the National Medical Ethics Committee of the Republic of Slovenia (reference number: KME 0120-357/2018/8).

CRediT authorship contribution statement

Sabina Ugovšek: conceptualization, methodology, investigation, data curation, writing – original draft preparation; Andreja Rehberger Likozar: conceptualization, methodology, investigation, writing – review and editing; Tina Levstek: data curation, writing – review and editing; Katarina Trebušak Podkrajšek: data curation, writing – review and editing, resources; Janja Zupan: data curation, writing – review and editing, visualization; Frieda Marka: data curation, writing – review and editing; Tamara Trimmel: data curation, writing – review and editing; Mira Brekalo: data curation, writing – review and editing; Walter Speidl: data curation, writing – review and editing; Philipp Hohensinner: data curation, writing – review and editing, resources, supervision; Patrick Haider: data curation, writing – review and editing, visualization; Miran Šebeštjen: conceptualization, methodology, investigation, data curation, writing – original draft preparation, resources, funding acquisition, project administration, supervision.

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Miran Sebestjen reports equipment, drugs, or supplies was provided by Slovenian Research Agency research programs P3-0308, P1-0420 and

P1-0170. None reports a relationship with None that includes:. None has patent None pending to None. None If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Graphical abstract was created using BioRender (https://biorender.com/, accessed on 26 January 2026).

Data availability

All data needed to evaluate the conclusions in the article are present in the article. Additional data related to this article are available upon reasonable request from the authors. The Major Resources Table can be found in the Supplemental Material.

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