Platelet function and activation markers in primary hypercholesterolemia treated with anti-PCSK9 monoclonal antibody: A 12-month follow-up

Platelet function and activation markers in primary hypercholesterolemia treated with PCSK9 monoclonal antibody: a 12-month follow-up.

Cristina Barale[1], Katia Bonomo[2], Chiara Frascaroli[2], Alessandro Morotti[1], Angelo Guerrasio[1], Franco Cavalot[2], Isabella Russo[1].

[1]Department of Clinical and Biological Sciences of Turin University, Orbassano, Turin (Italy),

[2]Metabolic Disease and Diabetes Unit, San Luigi Gonzaga Hospital, Orbassano, Turin (Italy).

Corresponding Author:

Dr Isabella Russo, PhD

Department of Clinical and Biological Sciences of the Turin University,

San Luigi Gonzaga Hospital,

Regione Gonzole, 10

I-10043 Orbassano (Turin), Italy

Phone: + 39 011 9026622 - Fax: + 39 011 9038639

e-mail: isabella.russo@unito.it

Word Counts
Abstract: 237
Text: 4064

Number of References: n.41

Figures: n.4

Tables: n.2

ABSTRACT

***Background and Aims*: In the association between hypercholesterolemia (HC) and thrombotic risk platelet hyper-reactivity plays an important role. The inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) to reduce plasma LDL-cholesterol merges as effective therapeutic strategy to prevent cardiovascular (CV) events. Aim of this study was to verify whether a treatment up to 12 months with the monoclonal antibodies (mAbs) anti-PCSK9 influences platelet function in primary HC.**

***Methods and Results*: In patients affected by primary HC (n=24), all on background of statin and 17 on acetyl salicylic acid (ASA), platelet function parameters were evaluated at baseline up to 12 months of treatment with the mAb anti-PCSK9 alirocumab or evolocumab.**

From baseline, the treatment with anti-PCSK9 mAbs: i) in ASA HC patients, significantly decreased platelet aggregation detected in platelet-rich plasma by light transmission aggregometry and in whole blood Platelet Function Analyzer-100 assay ; ii) in all HC patients, significantly decreased platelet membrane expression of CD62P and plasma levels of the *in vivo* platelet activation markers soluble CD40 Ligand, Platelet Factor-4, and soluble P-Selectin. Furthermore, CD62P expression, and sP-Selectin, PF-4, sCD40L levels significantly correlated with serum PCSK9.

***Conclusion*: Besides markedly lowering LDL-c levels, our results suggest that HC patients benefit from PCSK9 mAb treatment also for reducing platelet reactivity and increasing platelet sensitivity to the inhibitory effects of aspirin. These effects on platelets could play a role in the reduction of CV event incidence in patients treated with PCSK9-inhibitors.**

Keywords: platelets, hypercholesterolemia, PCSK9, acetyl salicylic acid.

INTRODUCTION

The correction of elevated circulating levels of low-density lipoprotein cholesterol (LDL-c) remains an unequivocal way to reduce cardiovascular (CV) risk [1] and strategies focusing in its prevention include lifestyle changes and new drugs. The inhibition of proprotein convertase subtilisin/kexin type 9 (PCSK9) to reduce plasma LDL-c is a new approach for the treatment of hypercholesterolaemia (HC) and emerges as effective promising therapeutic strategy to address the unmet clinical needs of achieving goal LDL-c levels for the majority of patients with high CV risk. PCSK9 is a major regulator of LDL-c levels as it promotes the degradation of hepatic LDL-c receptors, thus its inhibition causes an increase of LDL receptor activity and more circulating LDL-c is removed [2].

In Europe, alirocumab and evolocumab, two fully human anti-PCSK9 mAbs, in addition to diet and maximum tolerated statin therapy, are approved as agents available for use in adults with familial and non-familial HC or mixed dyslipidaemia who do not achieve target LDL-c with other lipid-lowering medication and in patients with statin intolerance [3]. Data from randomized controlled trials aimed at evaluating the impact of PCSK9 mAbs on CV outcomes showed clinical benefit for both evolocumab [4] and alirocumab [5] in patients at high risk. The association between HC and the development of a prothrombotic environment due, at least in part, to increased platelet biogenesis, turnover and activity is not new. Multiple mechanisms are involved in the increased platelet activation [6,7].. Activated platelets interact with endothelial cells of inflamed or atherosclerotic arteries and release platelet factors that trigger inflammatory reaction of endothelial cells and/or facilitate leukocyte–endothelial interactions. Given the fundamental role of platelets in mediating atherothrombosis, antiplatelet drugs play a pivotal role in preventing and treating arterial thrombosis. Among these, the cyclooxygenase-1 (COX-1) inhibitor acetylsalicylic acid (ASA) is a cornerstone of therapy in high CV risk patients. ASA blocks the production of thromboxane (TX) A_2 via the

irreversible inhibition of COX-1, thereby inhibiting TXA_2-induced platelet activation and aggregation, and provides major benefits in secondary prevention, reducing the relative risk of myocardial infarction by 25% [8]. However, in some individuals with HC the beneficial ASA effects may be restrained because of a reduced ability of aspirin to protect against thrombosis indicating that HC per se is likely to be linked to lower effects of ASA [9–11].

Statins have significantly reduced cardiovascular morbidity and mortality with platelet actions attributable not only to lipid-lowering properties but also to their pleiotropic effects [12–14] .

Similarly to statins, pleiotropic effects of PCSK9 inhibitors can be ascribed to their ability to reduce vascular events independently of lipid metabolism [15]. Indeed, PCSK9 inhibitors may directly influence haemostatic system given that a direct correlation between PCSK9 levels and high-on-treatment platelet reactivity [16] and effects on platelet activation [17] have been found. The interplay between anti-PCSK9 therapy and platelet function in HC has not been elucidated yet.

The objective of this study was to verify whether a treatment up to 12 months with anti-PCSK9 mAbs influences platelet function in primary HC.

MATERIALS AND METHODS

Chemicals

Collagen and arachidonic acid (AA) were purchased from Mascia Brunelli Spa (Monza, Milan, Italy). The other reagents, if not otherwise specified, were obtained from Sigma (St. Louis, MO, USA).

Population and blood collection

We conducted a prospective, single center cohort study of 24 patients with primary HC undergone treatment with anti-PCSK9 mAbs. The diagnosis of familial HC was established on the basis of clinical characteristics and laboratory parameters; criteria to identify familial HC firstly included the Dutch Lipid Clinic Network (DLCN) score [18] and patients with the clinical suspect of primary HC were then referred for genetic testing. The genetic diagnosis of familial HC were obtained by detecting promoter and coding DNA sequences and exon-intron boundaries regions of the LDL-receptor (LDLR), apolipoprotein B (APOB), PCSK9, apolipoprotein E (APOE) and signal-transducing adaptor protein 1 (STAP1) genes. The patients of this study were included in the LIPIGEN (Lipid TransPort Disorders Italian Genetic Network) network and genetic testing of the appropriate candidate genes were referred at one molecular diagnostic laboratory serving as nationwide DNA diagnostic center [19].

Chronic infection, systemic autoimmune disease, diabetes, obesity, history of alcohol or drug abuse, cancer or liver insufficiency were criteria of exclusion for enrolment in the study.

Patients were studied before and after being randomly allocated to receive either alirocumab (150 mg every two weeks) (n=19) or evolocumab (140 mg every two weeks) (n=5). Among them, 14 were taking ASA for previous cardiovascular events (100 mg/die). The regularity of ASA intake (100 mg/day) was based on the carefully obtained medical history. A normocholesterolemia (NC) group (n=21) served as controls.

All patients had received the maximum tolerated statin therapy before PCSK9 inhibitor administration. Informed consent was obtained before investigation and Ethic Committee of our Hospital approved the study.

A blood sample was collected before initiation of therapy (baseline) and after 2, 6 and 12 months (within 2 days from last injection). Blood samples were collected from the antecubital vein and all measurements were performed after a 12-h overnight fast. Laboratory parameters, including assessment of fasting glucose, total cholesterol, triglycerides, LDL-c, high-density lipoprotein cholesterol (HDL-c), platelets were measured using routine laboratory methods, performed by the central laboratory of our Hospital. Plasma and serum were stored at −70°C until the analysis of the other circulating parameters.

Platelet sample preparation

For studies concerning platelet function, a venous blood sample was withdrawn without stasis, and anticoagulated with 3.8% sodium citrate, pH 7.4 (vol/vol: 1/9) for aggregation studies in platelet-rich plasma (PRP) or with citrate-dextrose solution (ACD; v/v, 1/6) for experiments on washed platelets (WP). To prepare WP, the pellet was washed 2 times at 37 °C in HEPES-Na buffer (10 mmol/L HEPES Na, 140 mmol/L NaCl, 2.1 mmol/L MgSO4, 10 mmol/L d-glucose, pH 7.4).

Platelet aggregation studies

Light transmission aggregometry (LTA) was measured in PRP using an eight-channel aggregation system (Platelet Aggregation Profiler, Model PAP-8, BioData Corporation). Aggregation was stimulated by adenosine diphosphate (ADP) (10 µmol/l), collagen (4 mg/l) or AA (2 mmol/l). The aggregation was recorded for 5 min and the maximal aggregation expressed as maximum percentage change in LTA.

Platelet reactivity in high shear-stress conditions

Platelet adhesion/aggregation under high shear stress conditions was evaluated in whole blood (WB) samples by using Platelet Function Analyzer (PFA-100, Siemens Healthcare Diagnostic Products GmbH, Marburg, Germany) [20], which measures the time, named "closure time" (CT), needed to form a platelet plug within an aperture cut into a platelet-reactive membrane coated with collagen plus epinephrine (CEPI) or collagen plus ADP (CADP). PFA-100 test is FDA approved for detecting ASA responses within the CEPI cartridge [21] and subjects were considered poor responder to ASA if CT with CEPI cartridge was <193 seconds despite aspirin treatment, as suggested by the manufacturer.

CD62P expression

The expression of P-Selectin (CD62P) on platelet membrane was evaluated in the absence and in the presence of ADP (10 µmol/l), collagen (4 mg/l) or AA (100 µmol/l) for 8 min. Samples were processed as previously described [22]. Briefly, WP samples were incubated with fluorescein-conjugated mouse monoclonal anti-human P-selectin (CD62P) (R&D Systems) antibody or control antibody (isotype control). Allophycocyanin-conjugated mouse monoclonal anti-Human Integrin α2b/CD41 (R&D Systems) was used as positive control for platelet identification. CD62P expression was determined by using CyAn

Advanced Digital Processor Analyzer (Beckman Coulter, Brea, California, United States) and expressed as mean fluorescence intensity (MFI).

Assessment of circulating markers of platelet activation

The assessment of circulating markers of platelet activation consisted of the soluble CD40 Ligand (sCD40L), Platelet Factor (PF)-4 and soluble P-Selectin (sP-Selectin).

All these soluble markers were measured in plasma samples by employing the quantitative sandwich enzyme immunoassay technique from Cusabio Biotech Co. (College Park, MD, USA). Absorbance of products at 450 nm was proportional to the amount of analyte measured in the plasma sample.

Measurement of serum PCSK9 levels

To measure serum levels of PSCK9 a commercial kit (Human PCSK9 Simple Step Elisa kit, Abcam, Cambridge, UK) was used. The intra- and inter-assay coefficients of variation were estimated at 4.4% and 4.6%, respectively.

Statistical analysis

Of the 24 HC patients, three patients refused to continue the study after 8 weeks of treatment with anti-PCSK9 therapy. Thus, with respect to baseline, the comparison analyses were performed after 2 months for all patients (10 non-ASA, 14 on ASA) and after 6 and 12 months for 21 patients (7 non-ASA, 14 on ASA). For comparison between variables belonging to patients versus controls, unpaired Student t test for normally distributed data or Mann-Whitney U test for non-Gaussian data distribution were used. Results concerning PFA-100 have been evaluated with the nonparametric Wilcoxon signed rank test. Analysis of variance (ANOVA) for repeated measures with Bonferroni post hoc test or Wilcoxon signed-rank test,

as appropriate, were used to measure the within differences in platelet function and biochemical parameters in patients. Pearson's or Spearman correlation was used to examine the significance of correlation between variables, as appropriate. All statistical analyses were performed using SPSS 24.0 for Windows (SPSS Institute, Chicago, IL). All values in the text and figures are expressed as mean ± SD or median (range), considering their distributions. Results were significant when $p<0.05$.

RESULTS

The analysis performed for the genetic diagnosis showed that 18 patients were heterozygotes carriers of mutation in the LDLR gene and 1 patient was heterozygote carrier of mutation in the APOB gene. These mutations were classified as variants with clinical implications for familial HC. Five patients were not positive for the investigated mutations.

Effects of treatment with anti-PCSK-9 mAbs on serum lipid profile

Complete lipid profile and other clinical parameters in NC subjects and in the whole cohort of HC patients during the study are reported in Table 1.

In HC patients, the treatment with PCSK-9 inhibitors significantly lowered total cholesterol ($p<0.0001$), LDL-c ($p<0.0001$) after 2 months of treatment without further significant reduction. With respect to the PCSK9 inhibitor administrated, LDL-c levels, if compared with baseline values, were reduced by: i) 55±5% ($p<0.001$) after 2 months, 53±6% ($p<0.009$) after 6 months, and 55±6% ($p<0.01$) after 12 months with alirocumab; ii) 63±12% ($p<0.05$) after 2 months, 60±8% ($p<0.05$) after 6 months and 78±5% ($p<0.03$) after 12 months with evolocumab. Alirocumab and evolocumab did not significantly differ from each other for

their ability to reduce lipid parameters and did not change blood pressure, body mass index (BMI), HDL-c, glucose and platelet throughout the study.

Effects of treatment with anti-PCSK-9 mAbs on platelet aggregation

In comparison with NC, at baseline: i) HC patients on ASA showed lower aggregation to ADP ($p<0.0001$), collagen ($p<0.0001$) and AA ($p<0.0001$), thus indicating the aspirin effect on platelet aggregability, ii) non-ASA patients showed a greater aggregability to ADP ($p=0.004$) (Table 2). The effects of a treatment up to a one-year with anti-PCSK9 mAbs on platelet aggregation in patients stratified by ASA treatment are shown in Fig.1.

In non-ASA HC patients platelet aggregations to ADP (Fig.1A), collagen (Fig.1B) or AA (Fig.1C) did not significantly change during the study (versus baseline values, p=ns for each agonist at 2, 6, 12 months). In ASA HC patients significant reductions of platelet aggregation were observed starting at 2 months for collagen ($p=0.002$)(Fig.1B), and at 6 months in response to ADP ($p=0.01$)(Fig.1A) and AA ($p=0.03$)(Fig.1C). Noteworthy, the quantitative determination of platelet function triggered by AA, more than collagen and ADP, is specific for measuring COX-1 activity as TXA_2 formed by AA metabolism is crucial for amplification of platelet activation to drive secondary aggregation. In all ASA HC patients the significant reduction of LTA-AA after PCSK9-inhibitors treatment suggests a decrease of COX-1 activity.

To explore the PCKS inhibitor effects on platelet aggregation in high shear stress conditions, WB samples from HC patients were analysed by PFA-100. As shown in Table 2, at baseline, PFA-100 CEPI CT values did not differ in non-ASA HC patients in comparison with NC controls, whereas they were significantly higher in ASA HC patients ($p<0.0001$), as the expected consequence of aspirin intake. From the beginning of therapy, a 2-month treatment with anti-PCKS9 mAbs significantly increased PFA-100 CEPI CT (median from 160 to 300

seconds, p=008) in ASA HC patients (p=0.025), thus suggesting an improvement of the inhibitory effects of aspirin on platelet response (Fig.1D). PFA-100 CT values measured at 6 and 12 months did not significantly change with respect to 2 months.

As expected, PFA-100 CADP CT values did not change in all HC subjects throughout the study passing from 95.6±7 sec at baseline to 98.1±5 sec after 12 months of treatment with anti-PCSK9 mAbs.

Platelet aggregation evaluated as LTA or PFA-100 in ASA HC assigned to take alirocumab or evolocumab did not significantly differ each other at baseline and during the study.

Effects of treatment with anti-PCSK-9 mAbs on CD62P expression

Detection of CD62P (P-selectin, an α-granule membrane protein) on platelet surface was assessed to ascertain platelet activation *ex vivo* and in response to collagen and AA. At baseline, non-ASA HC patients, if compared with NC controls, showed platelet CD62P expression significantly higher both in the absence of stimulus (p=0.04) and with collagen (p<0.0001) or AA (p<0.0001). ASA HC patients did not differ from NC subjects for CD62P expression without stimulus or with collagen (ns for both) whereas they presented higher CD62P expression in response to AA (p=0.05) (Table 2).

The effects of treatment with PCSK-inhibitors on CD62P expression are shown in Fig.2. With respect to baseline, in platelets without stimulus CD62P expression significantly decreased after 2 months both in non-ASA (p=0.05) and ASA (p=0.002) (Fig.2A). The collagen-induced CD62P expression decreased starting at 2 months in non-ASA HC (p=0.04) and in ASA HC

patients (p=0.002) (Fig.2B). The CD62P expression stimulated by AA significantly decreased starting at 6 months in non-ASA (p=0.04) and at 2 months in ASA HC (p=0.006)(Fig.2C).

Effects of treatment with anti-PCSK-9 mAbs on "in vivo" platelet activation markers

Upon activation platelets secret their granule contents into circulation. Several markers of platelet activation such as sP-Selectin, PF4, sCD40L, have been identified to correlate with the presence of inflammation and atherosclerosis [23]. The effects of treatment with PCSK-inhibitors on these circulating markers in HC patients stratified by ASA treatment are shown in Fig.3. A significant reduction of sP-Selectin (Fig. 3A), PF-4 (Fig. 3B), and sCD-40L (Fig. 3C) levels was observd only after 12 months of therapy with anti-PCSK9 mAbs in both non-ASA (vs baseline: p=0.043, p=0.046, and p=0.01, respectively) and ASA (vs baseline: p=0.009, p=0.007, and p=0.003 respectively) patients.

Correlation analyses

Univariate linear regression analysis including all subjects at baseline revealed: i)significant correlations of LDL-c with: CD62P expression without (p=0.041, r=0.309) and after stimulation with collagen (p=0.004, r=0.428) or AA (p=0.001, r=493), PF-4 (p=0.018, r=0.355), sP-Selectin (p=0.046, r=0.303), and sCD40L (p=0.002, r=0.450); ii)significant correlation of PCSK9 levels with: i) total cholesterol (p=0.0001, r=0.648) and LDL-c (p=0.0001, r=0.645), ii) CD62P expression stimulated by AA (p=0.015, r=0.366), iii)sP-Selectin (p=0.017, r=0.359), PF-4 (p=0.05, r=0.294), and sCD40L (p=0.04, r=0.307) levels (Fig.4).

DISCUSSION

The present study, the first to evaluate platelet function to PCSK-9 inhibitors in patients with primary HC, demonstrated a reduction of platelet aggregability and activation after a treatment for 2 up to 12 months with the anti-PCSK-9 mAbs alirocumab or evolocumab. This improvement was evident in presence of concomitant therapy with aspirin suggesting an effective role of treatment with PCSK9-inhibitors in increasing the sensitivity to the antiplatelet effects of aspirin.

LDL-c is a known risk factor for cardiovascular diseases [24] and therapies aimed at lipid modification, such as statins, have been important tools for primary and secondary prevention of cardiovascular events. However, the low percentage of patients reaching a predefined LDL-c target and the significant residual risk despite the usage of statins use have justified the development of alternate approaches to lipid modification and PCSK-inhibitors are able in providing these patients with optimal lipid lowering [25]. Indeed, in our study the anti-PCSK9 mAbs alirocumab and evolocumab have demonstrated LDL-c lowering of more than 50-60% in patients with familial HC on background of statin therapy, with important effects on platelet aggregation and activation. It is known that HC primes platelets increasing platelet activation [26,27] and a correlation between cholesterol and parameters of platelet function has been also found in the present study. Although it is reasonable to suppose that the antiplatelet effect of PCSK-9 inhibitors can be mainly related to cholesterol reduction, it cannot be ruled out entirely that this effect goes beyond their lipid-lowering action. Actually, in our study HC showed higher PCSK9 levels than NC and positive correlations of PCSK9 levels were found not only with total and LDL-c but also with some parameters of platelet reactivity such as the platelet activation markers sCD40L, PF-4, and sP-Selectin. Given that a direct effect of PCSK9 in potentiating aggregation and activation induced by a weak agonist was found [17] and the relationship between PCSK9 and higher platelet reactivity was established also in other clinical settings [16,28,29], we can suppose that increased levels of

PCSK9 could directly influence platelet reactivity and PCSK9 inhibitors reduce PCSK9 direct stimulatory effects on platelets beyond their lipid-lowering properties.

When stratified for ASA intake, in ASA-patients reductions in platelet aggregation were significant starting at 2 months, whereas in non-ASA a trend to reduction was observed but not statistically significant. At baseline non-ASA patients differed from ASA takers for higher levels of total cholesterol and triglycerides. Despite the different lipid profile could play a role, it is likely that the low number of non-ASA patients who completed the study decreases our statistical power and it is certainly a limitation of this study.

The magnitude of on-ASA platelet reactivity was specifically quantified in our study by measuring LTA-AA and PFA-100 CT with CEPI cartridges. Platelet aggregation to AA is still considered as the historical gold standard of platelet function testing [30] for measuring ASA inhibition of COX-1 activity due to the very good agreement between aggregation to AA and serum TXB_2 measurement [31]. ASA HC patients of this study decreased the AA-triggered platelet aggregation and all reached LTA-AA values<20% after a 2-month treatment with PCSK9-inhibitors suggesting an improvement in the suppression of TXA_2 production. The reduced aggregability in PRP in ASA-patients after treatment with PCSK-inhibitors was confirmed by the increase of PFA-100 CEPI CT, a non-COX-1-dependent platelet function test used to determine response to ASA [31]. A prolonged CT with CEPI cartridges with normal CADP CT value is suggestive in our study for an increased platelet inhibition by aspirin, indicating a decrease of residual platelet aggregation among patients receiving aspirin after 2 months of treatment with PCSK9-inhibitors. Aspirin is a cornerstone of treatment in the secondary prevention of cardiovascular disease [32] and the lack of a platelet inhibition despite aspirin administration in high risk patients is associated with high risk for major cardiovascular events [9]. Although the phenomenon of depressed sensitivity of platelets to aspirin is most likely the result of poor compliance, clinical, biological, and genetic properties affecting platelet function [33], in HC an important role is played by mechanisms particularly

related to LDL-c. The accumulation of cholesterol in platelets markedly increases platelet biogenesis, turnover and activity [27] including a persistent platelet TXA_2 synthesis that can reduce the platelet suppressive effect of ASA. A study which aimed to investigate the relationship of PCSK9 and CV events in patients with atrial fibrillation showed a direct correlation between plasma PCSK9 levels and urinary 11-dehydro-thromboxane B_2 [29] suggesting PCSK9 as a factor able to interact with COX-1 activity. Despite urinary TXB_2 decreased in HC subjects upon their treatment with various lipid-lowering compounds [34,35], we cannot exclude that the reduction of circulating PCSK9 plays a role in increasing the inhibitory action of aspirin on COX-1 activity. The increased inhibition of COX-1 by suppression of PCSK9 could contribute, at least in part, to lower aggregability to AA and prolong CEPI PFA-100 CT observed in the ASA HC individuals of our study.

Our study has concerned platelets from individuals receiving ASA but not P2Y12 inhibitor as antiplatelet drug. Since an association of PCSK9 levels with platelet reactivity in patients affected by acute coronary syndrome treated with prasugrel or ticagrelor has been shown [16] and a correlation between PCSK9 serum levels and platelet reactivity parameters in HC was also found in our study, we can speculate that from the beginning of treatment with anti-PCSK9 mAbs an improvement of platelet responses in terms of reduced reactivity could also have been observed in HC patients receiving P2Y12 inhibitors. Of course, future studies are warranted to confirm this hypothesis.

CD62P cross-linking platelets and leukocytes is considered a pivotal mediator of platelet-leukocyte aggregate formation and adhesion to endothelium, thereby upregulating release of proinflammatory cytokines that link platelets to inflammation, thrombosis, and atherogenesis [36]. In both ASA- and non-ASA patients the treatment for 2 months with anti-PCSK-9 mAbs decreased the expression of CD62P in both the absence and presence of exogenous stimulus such as collagen or AA and these changes in comparison with baseline values were maintained until the end of the study. Differently from CD62P expression whose reduction occurred

rapidly after treatment with anti-PCSK9 mAbs, we observed that the decrease of plasma levels of the *in vivo* activation markers of platelet activation was delayed. In particular, a significant reduction from baseline of sCD40L, PF-4 and P-selectin levels was observed only at 12 months in both non-ASA and ASA HC groups. All patients had received the maximum tolerated statin therapy before anti-PCSK9 mAbs administration and it is known that statins exert CV effects influencing also platelet function [35,37–41]. This could slow a further decrease over time of circulating prothrombotic and atherogenic factors released mainly, but not exclusively, by platelets, thus requiring more time to reach a significant systemic reduction. Of course, given the limited sample size, further powered studies are warranted to confirm our findings and better explore the influence of anti-PCSK9 mAb on platelet function both in the absence and in the presence of anti-platelet drugs.

Collectively, our data first found that in primary HC the inhibition of PCSK-9 with alirocumab or evolocumab on a background of statin therapy, besides lowering LDL-c, improved platelet function decreasing platelet aggregability and activation and increasing platelet responsiveness to ASA. These findings suggest that HC patients benefit from a more intensive lowering of LDL-c levels also for improving platelet function and the use of the combination of PCSK9 inhibitors plus aspirin in secondary prevention of CVD could lead to a greatest reduction of CV event incidence.

ACKNOWLEDGMENTS

This work was in part supported by a grant from the Department of Clinical and Biological Sciences of Turin University (Ricerca Locale Ex-60%) to IR (RUSI_RILO_17), AM (MORA_RILO_16), and AG (GUEA_RILO_18).

CONFLICT OF INTEREST

None.

REFERENCES

[1] Authors/Task Force Members:, A.L. Catapano, I. Graham, G. De Backer, O. Wiklund, M.J. Chapman, H. Drexel, A.W. Hoes, C.S. Jennings, U. Landmesser, T.R. Pedersen, Ž. Reiner, G. Riccardi, M.-R. Taskinen, L. Tokgozoglu, W.M.M. Verschuren, C. Vlachopoulos, D.A. Wood, J.L. Zamorano, 2016 ESC/EAS Guidelines for the Management of Dyslipidaemias: The Task Force for the Management of Dyslipidaemias of the European Society of Cardiology (ESC) and European Atherosclerosis Society (EAS) Developed with the special contribution of the European Assocciation for Cardiovascular Prevention & Rehabilitation (EACPR), Atherosclerosis. 253 (2016) 281–344. doi:10.1016/j.atherosclerosis.2016.08.018.

[2] N.G. Seidah, Proprotein convertase subtilisin kexin 9 (PCSK9) inhibitors in the treatment of hypercholesterolemia and other pathologies, Curr. Pharm. Des. 19 (2013) 3161–3172.

[3] J.M. McKenney, Understanding PCSK9 and anti-PCSK9 therapies, J Clin Lipidol. 9 (2015) 170–186. doi:10.1016/j.jacl.2015.01.001.

[4] M.S. Sabatine, R.P. Giugliano, A.C. Keech, N. Honarpour, S.D. Wiviott, S.A. Murphy, J.F. Kuder, H. Wang, T. Liu, S.M. Wasserman, P.S. Sever, T.R. Pedersen, FOURIER Steering Committee and Investigators, Evolocumab and Clinical Outcomes in Patients with Cardiovascular Disease, N. Engl. J. Med. 376 (2017) 1713–1722. doi:10.1056/NEJMoa1615664.

[5] G.G. Schwartz, P.G. Steg, M. Szarek, D.L. Bhatt, V.A. Bittner, R. Diaz, J.M. Edelberg, S.G. Goodman, C. Hanotin, R.A. Harrington, J.W. Jukema, G. Lecorps, K.W. Mahaffey, A. Moryusef, R. Pordy, K. Quintero, M.T. Roe, W.J. Sasiela, J.-F. Tamby, P. Tricoci, H.D. White, A.M. Zeiher, ODYSSEY OUTCOMES Committees and Investigators, Alirocumab and Cardiovascular Outcomes after Acute Coronary Syndrome, N. Engl. J. Med. 379 (2018) 2097–2107. doi:10.1056/NEJMoa1801174.

[6] E.A. Podrez, T.V. Byzova, M. Febbraio, R.G. Salomon, Y. Ma, M. Valiyaveettil, E. Poliakov, M. Sun, P.J. Finton, B.R. Curtis, J. Chen, R. Zhang, R.L. Silverstein, S.L. Hazen, Platelet CD36 links hyperlipidemia, oxidant stress and a prothrombotic phenotype, Nat. Med. 13 (2007) 1086–1095. doi:10.1038/nm1626.

[7] S.J.A. Korporaal, I. Meurs, A.D. Hauer, R.B. Hildebrand, M. Hoekstra, H.T. Cate, D. Praticò, J.-W.N. Akkerman, T.J.C. Van Berkel, J. Kuiper, M. Van Eck, Deletion of the high-density lipoprotein receptor scavenger receptor BI in mice modulates thrombosis susceptibility and indirectly affects platelet function by elevation of plasma free cholesterol, Arterioscler. Thromb. Vasc. Biol. 31 (2011) 34–42. doi:10.1161/ATVBAHA.110.210252.

[8] Antithrombotic Trialists' Collaboration, Collaborative meta-analysis of randomised trials of antiplatelet therapy for prevention of death, myocardial infarction, and stroke in high risk patients, BMJ. 324 (2002) 71–86. doi:10.1136/bmj.324.7329.71.

[9] P.A. Howard, Aspirin resistance, Ann Pharmacother. 36 (2002) 1620–1624. doi:10.1345/aph.1C013.

[10] Steering Committee of the Physicians' Health Study Research Group, Final report on the aspirin component of the ongoing Physicians' Health Study, N. Engl. J. Med. 321 (1989) 129–135. doi:10.1056/NEJM198907203210301.

[11] J. Mayer, T. Eller, P. Brauer, E.M. Solleder, R.M. Schäfer, F. Keller, K. Kochsiek, Effects of long-term treatment with lovastatin on the clotting system and blood platelets, Ann. Hematol. 64 (1992) 196–201.

[12] D. Kearney, D. Fitzgerald, The anti-thrombotic effects of statins, J. Am. Coll. Cardiol. 33 (1999) 1305–1307.

[13] U. Rauch, J.I. Osende, J.H. Chesebro, V. Fuster, D.A. Vorchheimer, K. Harris, P. Harris, D.A. Sandler, J.T. Fallon, S. Jayaraman, J.J. Badimon, Statins and cardiovascular diseases: the multiple effects of lipid-lowering therapy by statins, Atherosclerosis. 153 (2000) 181–189.

[14] M.T. Santos, M.P. Fuset, M. Ruano, A. Moscardó, J. Valles, Effect of atorvastatin on platelet thromboxane A(2) synthesis in aspirin-treated patients with acute myocardial infarction, Am. J. Cardiol. 104 (2009) 1618–1623. doi:10.1016/j.amjcard.2009.07.039.

[15] F. Paciullo, S. Momi, P. Gresele, PCSK9 in Haemostasis and Thrombosis: Possible Pleiotropic Effects of PCSK9 Inhibitors in Cardiovascular Prevention, Thromb. Haemost. 119 (2019) 359–367. doi:10.1055/s-0038-1676863.

[16] E.P. Navarese, M. Kolodziejczak, M.-P. Winter, A. Alimohammadi, I.M. Lang, A. Buffon, G.Y. Lip, J.M. Siller-Matula, Association of PCSK9 with platelet reactivity in patients with acute coronary syndrome treated with prasugrel or ticagrelor: The PCSK9-REACT study, Int. J. Cardiol. 227 (2017) 644–649. doi:10.1016/j.ijcard.2016.10.084.

[17] M. Camera, L. Rossetti, S.S. Barbieri, I. Zanotti, B. Canciani, D. Trabattoni, M. Ruscica, E. Tremoli, N. Ferri, PCSK9 as a Positive Modulator of Platelet Activation, J. Am. Coll. Cardiol. 71 (2018) 952–954. doi:10.1016/j.jacc.2017.11.069.

[18] J.C. Defesche, P.J. Lansberg, M.A.W. Umans-Eckenhausen, J.J.P. Kastelein, Advanced method for the identification of patients with inherited hypercholesterolemia, Semin Vasc Med. 4 (2004) 59–65. doi:10.1055/s-2004-822987.

[19] M. Averna, A.B. Cefalù, M. Casula, D. Noto, M. Arca, S. Bertolini, S. Calandra, A.L. Catapano, P. Tarugi, LIPIGEN Group, Familial hypercholesterolemia: The Italian Atherosclerosis Society Network (LIPIGEN), Atheroscler Suppl. 29 (2017) 11–16. doi:10.1016/j.atherosclerosissup.2017.07.001.

[20] E.J. Favaloro, Clinical utility of the PFA-100, Semin. Thromb. Hemost. 34 (2008) 709–733. doi:10.1055/s-0029-1145254.

[21] P. Harrison, M.S. Robinson, I.J. Mackie, J. Joseph, S.J. McDonald, R. Liesner, G.F. Savidge, J. Pasi, S.J. Machin, Performance of the platelet function analyser PFA-100 in testing abnormalities of primary haemostasis, Blood Coagul. Fibrinolysis. 10 (1999) 25–31.

[22] C. Barale, R. Senkeev, F. Napoli, M. De Gobbi, A. Guerrasio, A. Morotti, I. Russo, Transferrin Saturation Inversely Correlates with Platelet Function, Thromb. Haemost. 119 (2019) 766–778. doi:10.1055/s-0039-1681061.

[23] Z.-T. Wang, Z. Wang, Y.-W. Hu, Possible roles of platelet-derived microparticles in atherosclerosis, Atherosclerosis. 248 (2016) 10–16. doi:10.1016/j.atherosclerosis.2016.03.004.

[24] J. Stamler, The problem of elevated blood cholesterol, Am J Public Health Nations Health. 50(3)Pt 2 (1960) 14–19. doi:10.2105/ajph.50.3_pt_2.14.

[25] K. Yadav, M. Sharma, K.C. Ferdinand, Proprotein convertase subtilisin/kexin type 9 (PCSK9) inhibitors: Present perspectives and future horizons, Nutr Metab Cardiovasc Dis. 26 (2016) 853–862. doi:10.1016/j.numecd.2016.05.006.

[26] S. Kaul, B.J. Waack, R.C. Padgett, R.M. Brooks, D.D. Heistad, Altered vascular responses to platelets from hypercholesterolemic humans, Circ. Res. 72 (1993) 737–743.

[27] N. Wang, A.R. Tall, Cholesterol in platelet biogenesis and activation, Blood. 127 (2016) 1949–1953. doi:10.1182/blood-2016-01-631259.

[28] S. Li, C.-G. Zhu, Y.-L. Guo, R.-X. Xu, Y. Zhang, J. Sun, J.-J. Li, The relationship between the plasma PCSK9 levels and platelet indices in patients with stable coronary artery disease, J. Atheroscler. Thromb. 22 (2015) 76–84. doi:10.5551/jat.25841.

[29] D. Pastori, C. Nocella, A. Farcomeni, S. Bartimoccia, M. Santulli, F. Vasaturo, R. Carnevale, D. Menichelli, F. Violi, P. Pignatelli, ATHERO-AF Study Group, Relationship of PCSK9 and Urinary Thromboxane Excretion to Cardiovascular Events in Patients With Atrial Fibrillation, J. Am. Coll. Cardiol. 70 (2017) 1455–1462. doi:10.1016/j.jacc.2017.07.743.

[30] P. Harrison, M. Lordkipanidzé, Testing platelet function, Hematol. Oncol. Clin. North Am. 27 (2013) 411–441. doi:10.1016/j.hoc.2013.03.003.

[31] P. Harrison, M.A. Bethel, I. Kennedy, R. Dinsdale, R. Coleman, R.R. Holman, Comparison of nine platelet function tests used to determine responses to different aspirin dosages in people with type 2 diabetes, Platelets. 30 (2019) 521–529. doi:10.1080/09537104.2018.1478402.

[32] R. De Caterina, G. Renda, Clinical use of aspirin in ischemic heart disease: past, present and future, Curr. Pharm. Des. 18 (2012) 5215–5223.

[33] G. Du, Q. Lin, J. Wang, A brief review on the mechanisms of aspirin resistance, Int. J. Cardiol. 220 (2016) 21–26. doi:10.1016/j.ijcard.2016.06.104.

[34] L. Puccetti, A.L. Pasqui, M. Pastorelli, G. Bova, M. Di Renzo, A. Leo, M. Cercignani, A. Palazzuoli, A. Auteri, F. Bruni, Platelet hyperactivity after statin treatment discontinuation, Thromb. Haemost. 90 (2003) 476–482. doi:10.1160/TH03-02-0111.

[35] C. Barale, C. Frascaroli, R. Senkeev, F. Cavalot, I. Russo, Simvastatin Effects on Inflammation and Platelet Activation Markers in Hypercholesterolemia, Biomed Res Int. 2018 (2018) 6508709. doi:10.1155/2018/6508709.

[36] Y.-Q. Ma, E.F. Plow, J.-G. Geng, P-selectin binding to P-selectin glycoprotein ligand-1 induces an intermediate state of alphaMbeta2 activation and acts cooperatively with extracellular stimuli to support maximal adhesion of human neutrophils, Blood. 104 (2004) 2549–2556. doi:10.1182/blood-2004-03-1108.

[37] I. Aoki, N. Aoki, K. Kawano, K. Shimoyama, A. Maki, M. Homori, A. Yanagisawa, M. Yamamoto, Y. Kawai, K. Ishikawa, Platelet-dependent thrombin generation in patients with hyperlipidemia, J. Am. Coll. Cardiol. 30 (1997) 91–96.

[38] U. Laufs, K. Gertz, P. Huang, G. Nickenig, M. Böhm, U. Dirnagl, M. Endres, Atorvastatin upregulates type III nitric oxide synthase in thrombocytes, decreases platelet activation, and protects from cerebral ischemia in normocholesterolemic mice, Stroke. 31 (2000) 2442–2449.

[39] N. Haramaki, H. Ikeda, K. Takenaka, A. Katoh, R. Sugano, S. Yamagishi, H. Matsuoka, T. Imaizumi, Fluvastatin alters platelet aggregability in patients with hypercholesterolemia: possible improvement of intraplatelet redox imbalance via HMG-

CoA reductase, Arterioscler. Thromb. Vasc. Biol. 27 (2007) 1471–1477. doi:10.1161/ATVBAHA.106.128793.

[40] F.Y. Ali, P.C.J. Armstrong, A.-R.A. Dhanji, A.T. Tucker, M.J. Paul-Clark, J.A. Mitchell, T.D. Warner, Antiplatelet actions of statins and fibrates are mediated by PPARs, Arterioscler. Thromb. Vasc. Biol. 29 (2009) 706–711. doi:10.1161/ATVBAHA.108.183160.

[41] P. Pignatelli, R. Carnevale, D. Pastori, R. Cangemi, L. Napoleone, S. Bartimoccia, C. Nocella, S. Basili, F. Violi, Immediate antioxidant and antiplatelet effect of atorvastatin via inhibition of Nox2, Circulation. 126 (2012) 92–103. doi:10.1161/CIRCULATIONAHA.112.095554.

Legends of Figures

Figure 1

Light transmission aggregometry (LTA) in response to ADP(A), collagen(B), arachidonic acid (AA)(C) and Platelet Function Analyzer (PFA)-100 assay with collagen/epinephrine (CEPI) cartridge in patients with primary hypercholesterolemia at baseline and after 2, 6, 12 months of treatment with anti-PCSK-9 mAbs. Patients were stratified in acetyl salicylic acid taking (ASA) or not (non-ASA).(A-C) Data are presented as mean±SD. *p < 0.05 compared with baseline values.(D) Data are shown as box plots range from the first to the third quartile; bold line in the boxes represents the median.

Figure 2

Flow cytometry assessment of the platelet marker CD62P without stimulus(A), with collagen(B), or arachidonic acid (AA)(C) in patients with primary hypercholesterolemia at baseline and after 2, 6, 12 months of treatment with anti-PCSK-9 mAbs. Data are presented as mean±SD. MFI:mean fluorescence intensity. *p < 0.05 compared with baseline values.

Figure 3

Plasma levels of soluble P-Selectin (sP-Selectin), Platelet Factor-4 (PF-4), and soluble CD40 Ligand (sCD40L) in patients with primary hypercholesterolemia at baseline and after 2, 6, 12 months of treatment with anti-PCSK-9 mAbs. Data are presented as mean±SD. *p < 0.05 compared with baseline values.

Figure 4

Univariate linear regression analysis at baseline between proprotein convertase subtilisin/kexin type 9 (PCSK9) levels and platelet parameters in subjects with normocholesterolemia (open triangle) and hypercholesterolemia (black circle). TC:total cholesterol; LDL-C:low-density lipoprotein cholesterol; LTA:light transmission aggregometry; Coll:collagen; AA:arachidonic acid; PFA:Platelet Function Analyzer; CEPI: collagen/epinephrine; MFI:mean fluorescence intensity; sP-Sel:soluble P-Selectin; PF:platelet factor; sCD40L:soluble CD40 Ligand.

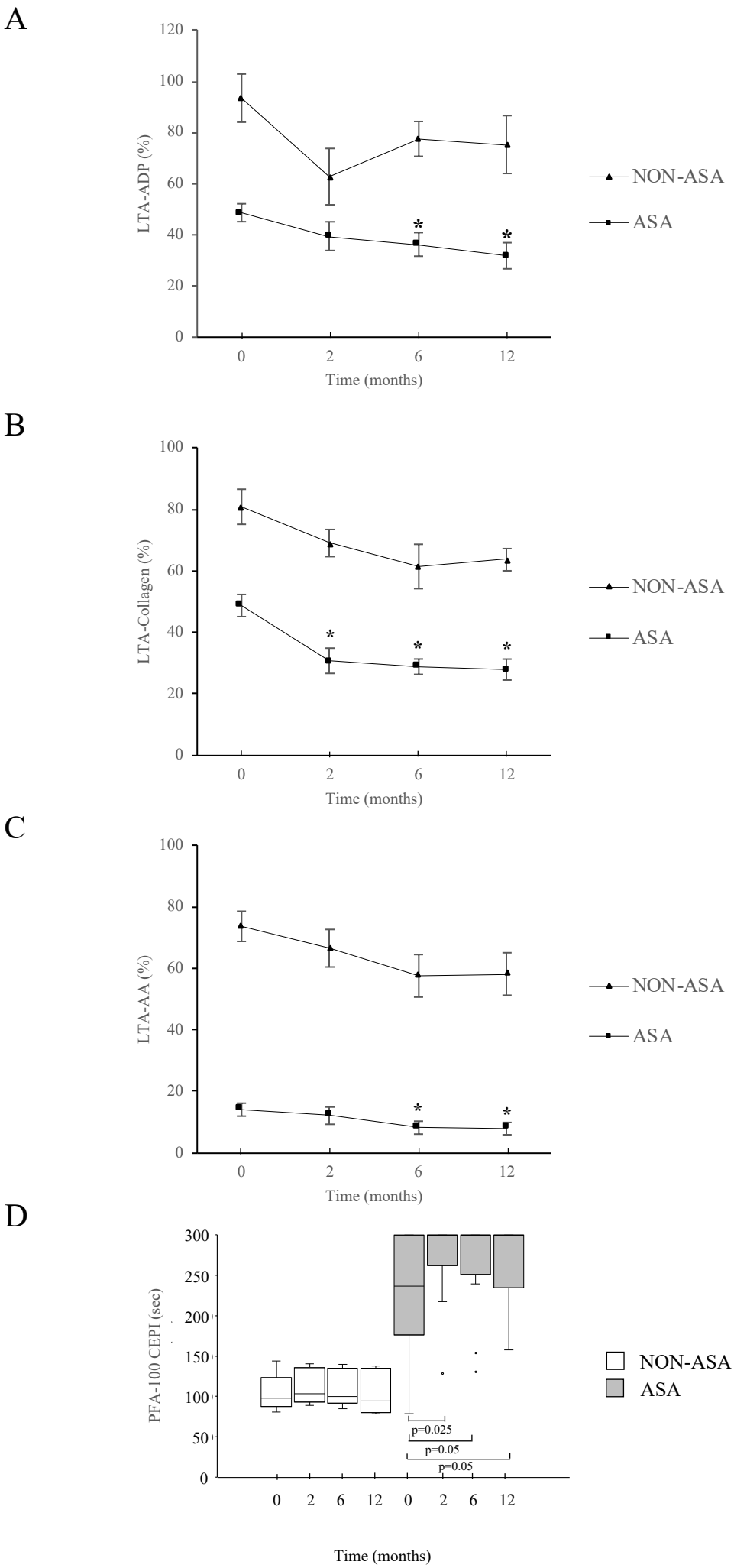
A
LTA-ADP (%)
120
100
80
60
40
20
0
0
2
6
12
Time (months)
*
*
NON-ASA
ASA
B
LTA-Collagen (%)
100
80
60
40
20
0
0
2
6
12
Time (months)
*
*
*
NON-ASA
ASA
C
LTA-AA (%)
100
80
60
40
20
0
0
2
6
12
Time (months)
*
*
NON-ASA
ASA
D
PFA-100 CEPI (sec)
300
250
200
150
100
50
0
0
2
6
12
0
2
6
12
Time (months)
p=0.025
p=0.05
p=0.05
NON-ASA
ASA

Figure 1

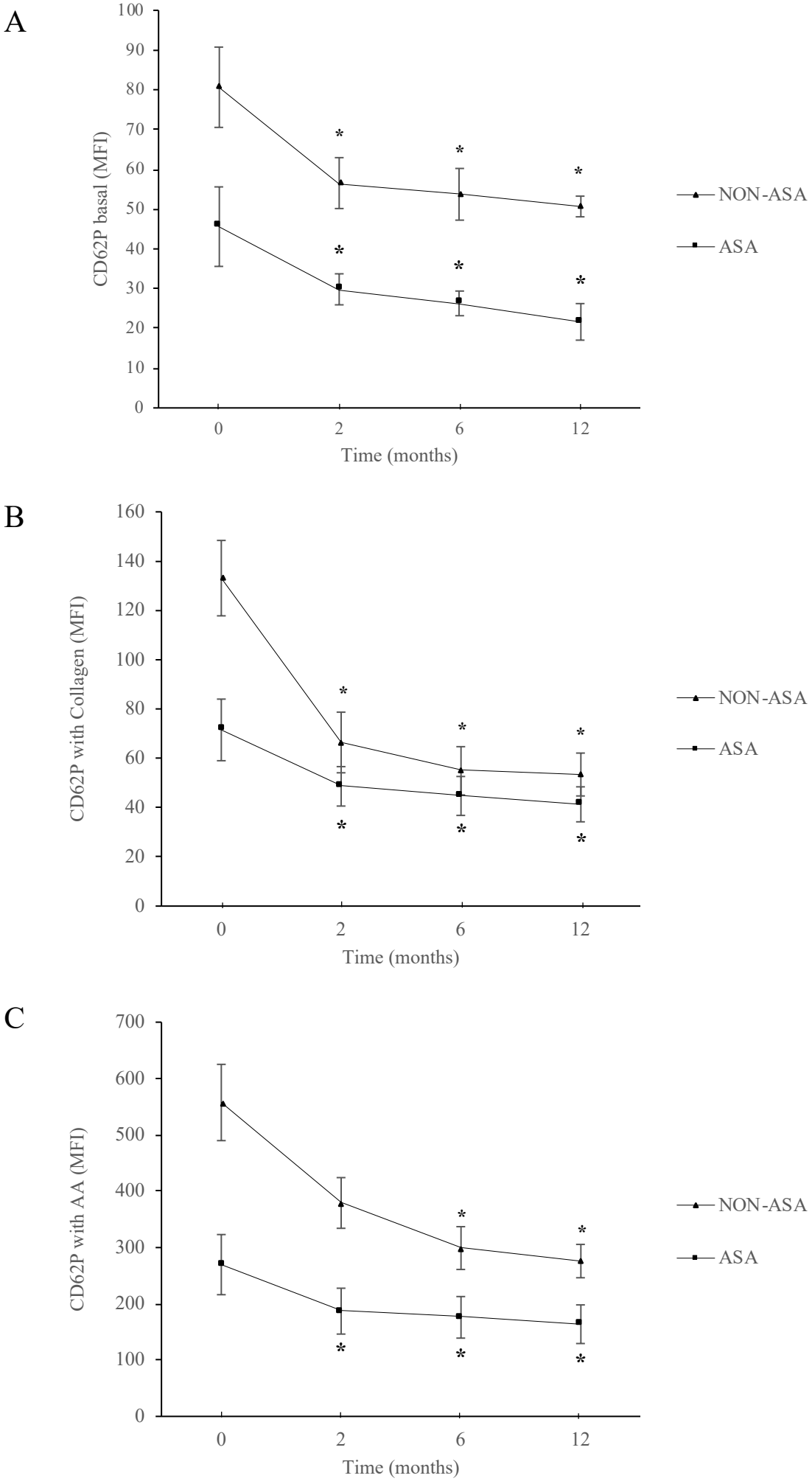
A
CD62P basal (MFI)
100
90
80
70
60
50
40
30
20
10
0
0
2
6
12
Time (months)
NON-ASA
ASA
B
CD62P with Collagen (MFI)
160
140
120
100
80
60
40
20
0
0
2
6
12
Time (months)
NON-ASA
ASA
C
CD62P with AA (MFI)
700
600
500
400
300
200
100
0
0
2
6
12
Time (months)
NON-ASA
ASA

Figure 2

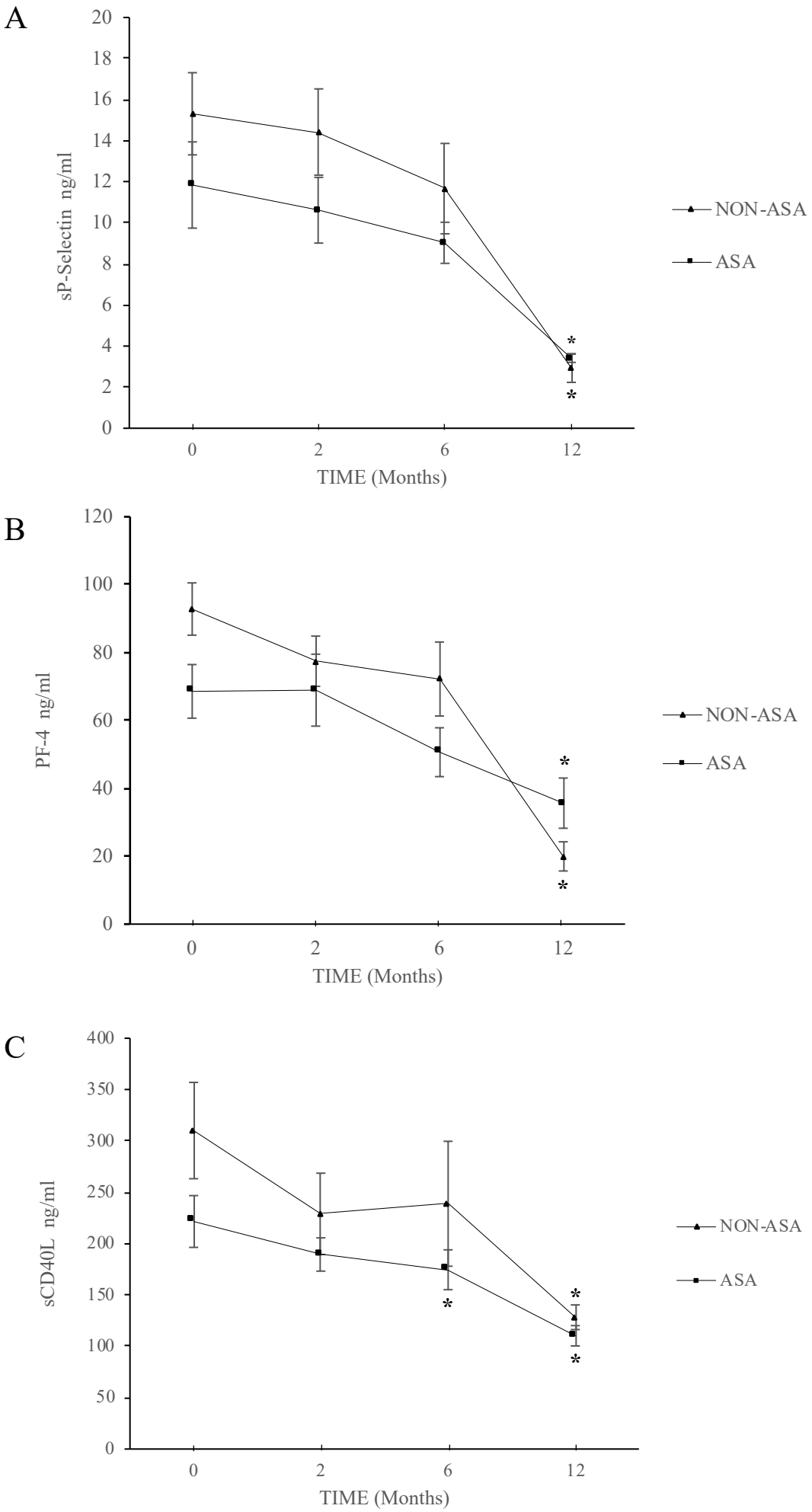
A
sP-Selectin ng/ml
20
18
16
14
12
10
8
6
4
2
0
0
2
6
12
TIME (Months)
NON-ASA
ASA
*
*
B
PF-4 ng/ml
120
100
80
60
40
20
0
0
2
6
12
TIME (Months)
NON-ASA
ASA
*
*
C
sCD40L ng/ml
400
350
300
250
200
150
100
50
0
0
2
6
12
TIME (Months)
NON-ASA
ASA
*
*
*

Figure 3

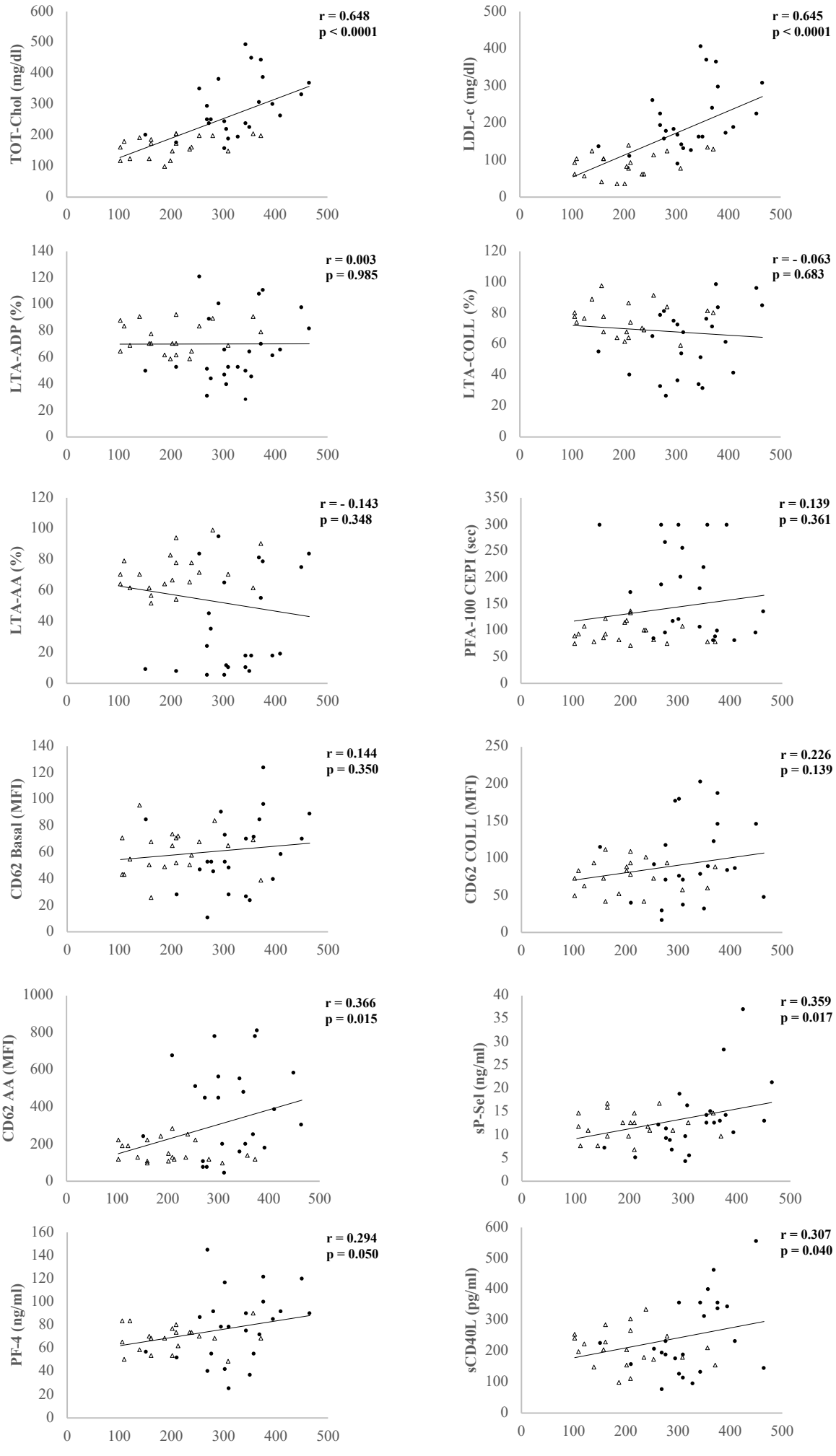
TOT-Chol (mg/dl)
r = 0.648
p < 0.0001
LDL-c (mg/dl)
r = 0.645
p < 0.0001
LTA-ADP (%)
r = 0.003
p = 0.985
LTA-COLL (%)
r = - 0.063
p = 0.683
LTA-AA (%)
r = - 0.143
p = 0.348
PFA-100 CEPI (sec)
r = 0.139
p = 0.361
CD62 Basal (MFI)
r = 0.144
p = 0.350
CD62 COLL (MFI)
r = 0.226
p = 0.139
CD62 AA (MFI)
r = 0.366
p = 0.015
sP-Sel (ng/ml)
r = 0.359
p = 0.017
PF-4 (ng/ml)
r = 0.294
p = 0.050
sCD40L (pg/ml)
r = 0.307
p = 0.040
Serum PCSK9 (ng/ml)
Serum PCSK9 (ng/ml)

Figure 4