Thrombosis Research 180 (2019) 74–85

Contents lists available at ScienceDirect

Thrombosis Research

journal homepage: www.elsevier.com/locate/thromres

Full Length Article

Hypercholesterolemia impairs the Glucagon-like peptide 1 action on platelets: Effects of a lipid-lowering treatment with simvastatin

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ARTICLE INFO

Keywords:
Glucagon-like peptide 1
Platelets
Nitric oxide
Hypercholesterolemia
Reactive oxygen species
Simvastatin

ABSTRACT

Background: The incretin hormone Glucagon-like peptide 1(GLP-1) plays a pivotal role in maintaining glucose homeostasis with effects also on the cardiovascular system. GLP-1 influences platelet functions by increasing the inhibitory action of nitric oxide (NO) and reducing oxidative stress. To date, the role of hypercholesterolemia (HyC) on platelet GLP-1 effects needs to be elucidated.

Methods: Forty-five subjects with primary HyC and twenty normocholesterolemic controls (NoC) were enrolled. In platelets from all subjects, the native GLP-1 (7-36), the truncated GLP-1 (9-36) and the GLP-1 analogue Liraglutide were evaluated in their ability to interfere with the activation of NO/PKG/VASP, PI-3K/Akt e MAPK/ERK-1/2 pathways and oxidative stress. Furthermore, in HyC subjects the role of a lipid-lowering therapy with statin on GLP-1 related peptide effects on platelet function was evaluated.

Results: Unlike in NoC, in platelets from HyC subjects the GLP-1 related peptides GLP-1 (7-36), GLP-1 (9-36) and Liraglutide all failed to: i) increase the antiaggregating effects of NO and the NO-induced VASP-ser239 phosphorylation, ii) decrease phosphorylation levels of Akt and ERK-2 and iii) reduce reactive oxygen species (ROS) generation. The treatment with simvastatin (40 mg/die) in HyC (n = 18) significantly reduced total and LDL cholesterol levels, platelet aggregability/activation, ROS production and NO action but did not modify platelet sensitivity to the GLP-1 effects.

Conclusion: Collectively, these results indicate that hypercholesterolemia per se is characterized by a resistance to GLP-1 effects on platelets and this impairment is not corrected by treatment with simvastatin.

1. Introduction

The incretin hormone Glucagon-like peptide 1 (GLP-1) plays a crucial role in maintaining glucose homeostasis and influences cardiovascular system [1]. Secreted as GLP-1(7-36) amide, it induces the increase of glucose-stimulated insulin secretion after binding to a specific GLP-1 receptor (GLP-1R). Since GLP-1(7-36) is rapidly degraded by the enzyme dipeptidyl peptidase (DPP)-4, the main GLP-1 metabolite in vivo is GLP-1(9-36) which does not interact with GLP-1R [2]. Despite GLP-1R agonists being an important class of drugs with a well-established efficacy and safety profile in patients with type 2 diabetes mellitus, not all tested GLP-1R agonists have been shown to reduce cardiovascular events [3–6].

The ability of GLP-1 related peptides to interfere with the platelet function has so far been demonstrated by few works [7,8]. The activation of cyclic adenosine monophosphate (cAMP)/protein kinase cAMP-dependent (PKA) pathway and GLP-1R are involved in the platelet effects of the GLP-1R agonist exenatide which inhibits aggregation and thrombus formation under flow conditions in human and mouse whole blood [7]. In our previous paper, we showed that platelet exposure to GLP-1 influences platelet function by reducing oxidative stress, increasing the inhibitory effects of the nitric oxide (NO)/cyclic guanosine monophosphate (cGMP)/protein kinase cGMP-dependent (PKG) pathway and decreasing the activation of phosphatidyl inositol 3-kinase (PI3K) and mitogen-activated protein kinase (MAPK) pathways through mechanisms independent of GLP-1 binding to GLP-1R which is normally expressed on platelet membrane [8]. Noteworthy, these GLP-1 effects on platelets are not mediated by cAMP signallling [8] which on the contrary is involved in the antiaggregating effect of GLP-1 when platelets are exposed at much higher concentrations of

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https://doi.org/10.1016/j.thromres.2019.06.010
Received 8 January 2019; Received in revised form 31 May 2019; Accepted 14 June 2019
Available online 15 June 2019

GLP-1R agonists [9]. The protective GLP-1 effects on platelets induce to hypothesise that a reduced action or synthesis of GLP-1 in some disease states may contribute to platelet hyperreactivity.

Platelets are deeply involved not only in haemostasis but also in the pathogenesis of atherosclerosis and thrombi formation and an impairment of NO action on platelets plays a pivotal a role in platelet hyperreactivity [10]. NO is a potent inhibitor of platelet adhesion to the subendothelium and their subsequent aggregation [11,12]. Atherosclerosis, and diseases which predispose to atherosclerosis such as hypercholesterolemia (HyC) and diabetes, are characterized by altered activity of platelets [10]. In the early stages of the disease reduced endogenous NO would leave the endothelium vulnerable to increased leukocyte diapedesis and increase the possibility of low-density lipoprotein (LDL) oxidation in the subendothelial space. In the final stages, loss and/or impaired NO action could induce platelet activation, leading to thrombosis and myocardial infarction. Hence reduced NO bioavailability could contribute to the progression of atherosclerosis at all stages of the disease process [13]. Currently, the foremost mechanism for the loss of bioavailable NO is thought to be NO scavenging by reactive oxygen species (ROS). Thus, in disease states such as HyC and diabetes, where ROS production is increased, NO metabolism is dysregulated. Therefore, an increase of bioavailable NO, either through endothelial or platelet production, is a critical determinant of platelet function, and this is perturbed in HyC.

Statins or 3-hydroxy-methylglutaryl coenzyme A reductase inhibitors have been used for 30 years to prevent coronary artery diseases and their protective cardiovascular effects are ascribed not only to reduced cholesterol levels but also to a number of additional effects, including antithrombotic effects [14,15]. Many studies have demonstrated antiplatelet activity of statins [16–19] with effects on a wide range of markers of inflammation and atherothrombosis [19]. However, no information is available on the influence of statin therapy on platelet GLP-1 effects.

The present study was designed to examine in primary HyC the ability of GLP-1 to influence in vitro the inhibitory effects of NO, signalling molecules of PI3K and MAPK pathways stimulated by pro-aggregating agents and ROS production. We tested the effects of the native GLP-1(7-36), of the N-terminally truncated GLP-1(9-36) form and of the GLP-1 analogue Liraglutide on platelets also after a lipid-lowering treatment for 3 months with simvastatin.

2. Materials and methods

2.1. Chemicals

Collagen and AA were purchased from Mascia Brunelli Spa (Monza, Milan, Italy). GLP-1(7-36), GLP-1(9-36) were obtained from DBA Italia Srl (Segrate, Milan, Italy). Liraglutide (VICTOZA®) was obtained from Novo Nordisk, Denmark. The sources of the specific antibodies are shown in the different sections. The other reagents were obtained from Sigma (St. Louis, MO, USA).

2.2. Subjects and platelet preparation

The study firstly comprised 44 patients (18 men/26 women; age: 51 ± 12 years) with primary HyC. They did not have a family history of diabetes mellitus and were otherwise healthy on the basis of medical history, physical examination and standard diagnostic procedures. In particular, they did not present arterial hypertension, impaired fasting glucose or impaired glucose tolerance measured by the oral glucose tolerance test (OGTT), congestive heart failure, previous peripheral or coronary or cerebral ischemic vascular diseases, endocrine diseases (including hypothyroidism), renal, hepatic or hepatobiliary diseases, myopathic or haemostatic disorders. Five patients were smokers (less than seven cigarettes/day). From the study, we also excluded patients on treatment with hypolipidemic, and antiplatelet, anticoagulant or profibrinolytic drugs in the previous two weeks. Twenty subjects with normocholesterolemia (NoC) (12 men/8 women; age: 51 ± 8 years) served as controls.

Furthermore, HyC patients were randomized to be treated with diet plus simvastatin 40 mg/die for three months ($n = 18$) or diet without pharmacological intervention ($n = 26$). All patients followed a low-fat diet close to the Adult Treatment Panel (ATP) III guidelines (7% energy from saturated fat and 200 mg dietary cholesterol per day).

The study was conducted in accordance with the Declaration of Helsinki and the protocol was approved by the Ethics Committee of San Luigi Gonzaga Hospital (Project identification code 89/2013). All participants authorized data use for investigational purpose by signed informed consent.

Fasting venous blood samples were collected by venepuncture into a plastic tube containing 3.8% trisodium citrate (v/v:1/9) or citrate-dextrose solution (ACD; v/v:1/6). Platelet-rich plasma (PRP) was obtained by using Platelet Function Centrifuge (BioData Corporation, Horsham, PA, USA). To prepare washed platelets (WP), ACD-anticoagulated PRP was processed as previously described [20].

2.3. Platelet aggregation studies

Platelet aggregation studies were carried out in PRP by following light-scattering changes using an eight-channel aggregation system (Platelet Aggregation Profiler, Model PAP-8, BioData Corporation). Platelet aggregation tests were carried out after a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) with or without the NO-donor sodium nitroprusside (SNP) (5μmol/l, 5 min) and induced by adenosine diphosphate (ADP) (5 μmol/l), arachidonic acid (AA) (0.5 mmol/l) or collagen (2 mg/l). Each aggregation test was recorded for 5 min and reported as maximal aggregation.

2.4. Signalling transduction molecule detection

WP were exposed for 15-min to GLP-1(7-36), GLP-1(9-36) or Liraglutide, then stimulated by SNP (5 μmol/l, 5 min) to evaluate the NO-induced PKG activation through detection of Vasodilator-Stimulated Phosphoprotein (VASP) phosphorylation at ser-239.

To evaluate GLP-1 influence on PI3K and MAPK pathways activation, WP were preincubated for 15-min with GLP-1 (7-36), GLP-1 (9-36) or Liraglutide, then stimulated for 8 min by collagen (2 mg/l) or AA (100 μmol/l). Then, samples were centrifuged, pellets dissolved in Laemmli buffer (1% SDS, 0.1% Triton X-100, 10 mmol/L Tris-HCl, pH 7.4, supplemented with protease inhibitors) and processed as previously described [20]. Membranes were then incubated with the following antibodies: mouse anti-Akt (1:1000), rabbit anti-phosphoAkt-Ser473 (1:1000), mouse anti-phosphoERK-1/2-Tyr204 (1:1000) and rabbit anti-VASP (1:1000) (Santa Cruz Biotechnology, Dallas, TX, USA), rabbit anti-ERK-2 (1:1000) (R&D Systems, Minneapolis, MN, USA), mouse anti-phosphoVASP-ser-239 (1:1000) (Calbiochem–Merck Millipore, Darmstadt, Germany). As secondary antibodies we used goat anti-mouse (1:1000) (Santa Cruz Biotechnology) and goat anti-rabbit (1:10000) (Cell Signaling, Danvers, MA, USA).

2.5. ROS production

Intracellular ROS were evaluated in WP by using 2',7'-dichlorofluorescein diacetate (DCF-DA) oxidized by H_2O_2 to the highly fluorescent DCF [21]. WP (6×10^7 ml^{-1}) were exposed to 10 μmol/l DCF-DA and incubated for 15 min with GLP-1(7-36), GLP-1(9-36) or Liraglutide. AA (100 μmol/l) was added just before measuring fluorescence.

Fluorescence was measured over a 60-min period at 1-min intervals using a plate fluorometer (GloMax-Multi Detection System, Promega Corporation, Madison, WI, USA) fitted with 490 nm excitation and 520 nm emission filters. Fluorescence per minute was first calculated

Table 1
Characteristics of the investigated subjects.

	NoC	HyC	*p*-Value	HyC No Simva before	HyC No Simva after	p-Value	HyC Simva before	HyC Simva after	p-Value
N(Male/Female)	20 (12/8)	44 (18/26)		26 (11/15)			18 (7/11)		
Age (years)	51 ± 8	51 ± 12	0.887	46 ± 12			57 ± 10[#]		
BMI (kg/m^2)	24 ± 1	25 ± 3	0.882	24 ± 3	24 ± 3	0.987	25 ± 4	25 ± 4	0.493
TC (mg/dl)	169 ± 17	273 ± 47	< 0.0001	262 ± 41	263 ± 36	0.374	288 ± 51[%]	206 ± 37[^]	< 0.0001
HDL-C (mg/dl)	57 ± 13	63 ± 19	0.241	65 ± 21	62 ± 16	0.075	59 ± 15	56 ± 14	0.431
TG (mg/dl)	117 ± 26	163 ± 84	0.021	149 ± 69	142 ± 62	0.989	183 ± 101	150 ± 120	0.145
LDL-C (mg/dl)	88 ± 24	182 ± 40	< 0.0001	171 ± 35	173 ± 30	0.747	198 ± 43[*]	150 ± 120,	< 0.0001
FG (mg/dl)	88 ± 5	91 ± 10	0.505	91 ± 9	89 ± 7	0.115	91 ± 12	90 ± 10	0.710
SBP/DBP (mm Hg)	120 ± 15/79 ± 7	121 ± 18/82 ± 7	0.829/0117	120 ± 23/82 ± 6	119 ± 20/83 ± 9	0.868/0.927	124 ± 8/82 ± 4	123 ± 10/81 ± 7	0.742/0.602
PLTs(10^3/μl)	259.7 ± 58	254.7 ± 48	0,951	258,0 ± 52	258,3 ± 44	0,890	252,0 ± 42	250,0 ± 37	0,509
MPV (fl)	7.9 ± 0.8	8.1 ± 0.7	0,411	8.1 ± 0.9	8.1 ± 0.7	0,606	8.4 ± 0.9	8.3 ± 0.9	0,520

Data are presented as mean ± SD; BMI: body mass index; TC: total cholesterol; HDL: high-density lipoprotein; TG: triglycerides; LDL: low-density lipoprotein; FG: fasting glucose; PLTs: platelets; MPV: mean platelet volume; SBP, systolic blood pressure; DBP, diastolic blood pressure. Unpaired or paired Student's *t*-test or Mann Whitney U or Wilcoxon test were used as appropriate. *a*: p = 0.002 vs no simvastatin before; *b*: p = 0.04 vs no simvastatin before, *c* = p < 0.0001 vs no simvastatin after, *d* = p = 0.03 vs no simvastatin before, *e*: p < 0.0001 vs no simvastatin after.

for each sample, then ROS production was expressed as DCF fluorescence.

2.6. Statistical analysis

Data are expressed as mean ± SD. Normality of data was checked using Shapiro–Wilk test. Continuous data was examined using parametric analyses performed by Student's *t*-test for paired and unpaired data or ANOVA followed by Bonferroni correction for multiple comparisons. Data with a non-Gaussian distribution were analysed using the Mann–Whitney *U* test and Wilcoxon signed-rank test, as appropriate. All tests were two-tailed and only values lower than 0.05 were regarded as statistically significant. All analyses were performed with SPSS v.24.

3. Results

In Table 1 clinical characteristics of NoC and HyC subjects at baseline and HyC subjects before and after treatment with diet alone or diet plus simvastatin (40 mg/die) were summarized. The levels of total cholesterol ($p < 0.0001$), LDL cholesterol ($p < 0.0001$) and triglycerides ($p = 0.021$) at baseline were higher in HyC ($n = 44$) than in NoC ($n = 20$).

In HyC subjects treated with simvastatin ($n = 18$), but not in those with diet alone ($n = 26$), total and LDL-cholesterol ($p < 0.0001$, for both) levels were significantly decreased after three months of treatment.

3.1. GLP-1 influence on the antiaggregating effect of SNP

PRP samples from HyC and NoC were exposed to the NO-donor SNP both in the absence and in the presence of GLP-1 (7-36), GLP-1 (9-36) and Liraglutide and aggregation to agonists measured as percentage of light-scattering changes. Platelet exposure to SNP significantly decreased platelet aggregation to ADP, collagen, or AA in both HyC and NoC (Fig. 1). However, the preincubation with GLP-1 related peptides significantly enhanced the antiaggregating effect of SNP in NoC but not in HyC patients. In particular, as compared with basal values, in the presence of SNP: i) in NoC, the fold reduction of platelet aggregation to ADP changed from 1.7 to 2.3, 2.3, and 2.4 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively ($p < 0.0001$ for each); in HyC, from 1.7 to 1.8, 1.9, and 1.8 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively (p = ns for each) (Fig. 1A); ii) in NoC, the fold reduction of platelet aggregation to collagen changed from 1.8 to 2.4, 2.6, and 2.6 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively ($p < 0.0001$ for each); in HyC, from 1.5 to 1.6, 1.7, and 1.7 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively (p = ns for each) (Fig. 1B); iii) in NoC, the fold reduction of platelet aggregation to AA changed from 1.5 to 2.1, 2.2, and 2.1 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively ($p < 0.0001$ for each); in HyC, from 1.6 to 1.8, 1.7, and 1.8 with GLP-1(7-36), GLP-1(9-36) and Liraglutide, respectively (p = ns for each) (Fig. 1C). In comparison with values at baseline, after simvastatin treatment platelets from HyC subjects ($n = 18$) showed: i) reduced aggregability to ADP (maximal aggregation from 84% ± 6% to 74% ± 4%, $p < 0.0001$) (Fig. 2A), collagen (maximal aggregation from 90% ± 4% to 84% ± 3%, $p = 0.013$) (Fig. 2B), and a trend to reduction to AA (maximal aggregation from 84% ± 5% to 75% ± 3%, $p = 0.08$) (Fig. 2C); ii) increased antiaggregating effects of SNP, since the fold reduction passed from 1.7 to 2.3, ($p = 0.003$) with ADP, from 1.5 to 2.0 ($p = 0.001$) with collagen and from 1.5 to 1.8 (p = 0.01) with AA. However, the preincubation with GLP-1 (7-36), GLP-1 (9-36) or Liraglutide did not influence the inhibitory effects of SNP on platelet aggregation to each agonist (versus SNP alone for each peptide, p = ns). In the no simvastatin group ($n = 26$), no significant change was observed in platelet response to SNP with or without GLP-1 related peptides. In all investigated subjects,

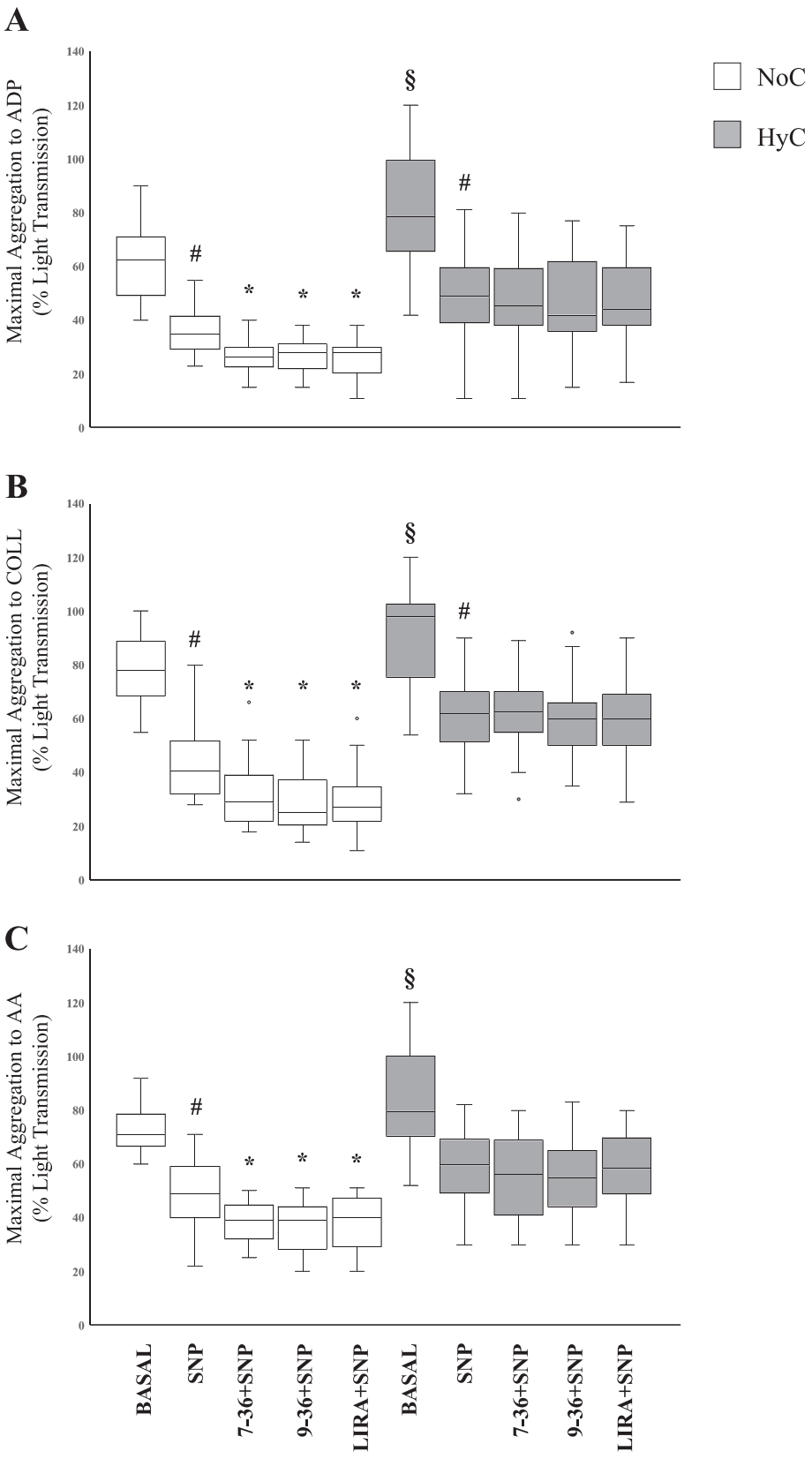


Fig. 1. Effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on platelet aggregation induced by ADP (5 μmol/l) (panel A), collagen (2 mg/l) (panel B) and arachidonic acid (AA) (100 μmol/l) (panel C) in the presence of sodium nitroprusside (SNP) (5 μmol/l) in normocholesterolemia (NoC) ($n = 20$) and hypercholesterolemia (HyC) ($n = 44$). Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range; open circles: outliers. Significance of intra-group: 1-way repeated measures ANOVA ($p < 0.0001$) followed by Bonferroni test: #$p < 0.05$ versus basal, *p < 0.05 vs SNP alone. Differences between NoC and HyC was estimated by *t*-test Student: §$p < 0.002$ versus NoC basal.

platelet exposure to GLP-1 (7-36), GLP-1 (9-36) or Liraglutide did not influence platelet aggregation to ADP, collagen or AA alone (data not shown).

3.2. GLP-1 effects on the SNP-induced VASP-ser239 phosphorylation

WP samples from HyC and NoC were stimulated by SNP both in the absence and presence of GLP-1 related peptides and pVASP-ser239 levels evaluated by western blot.

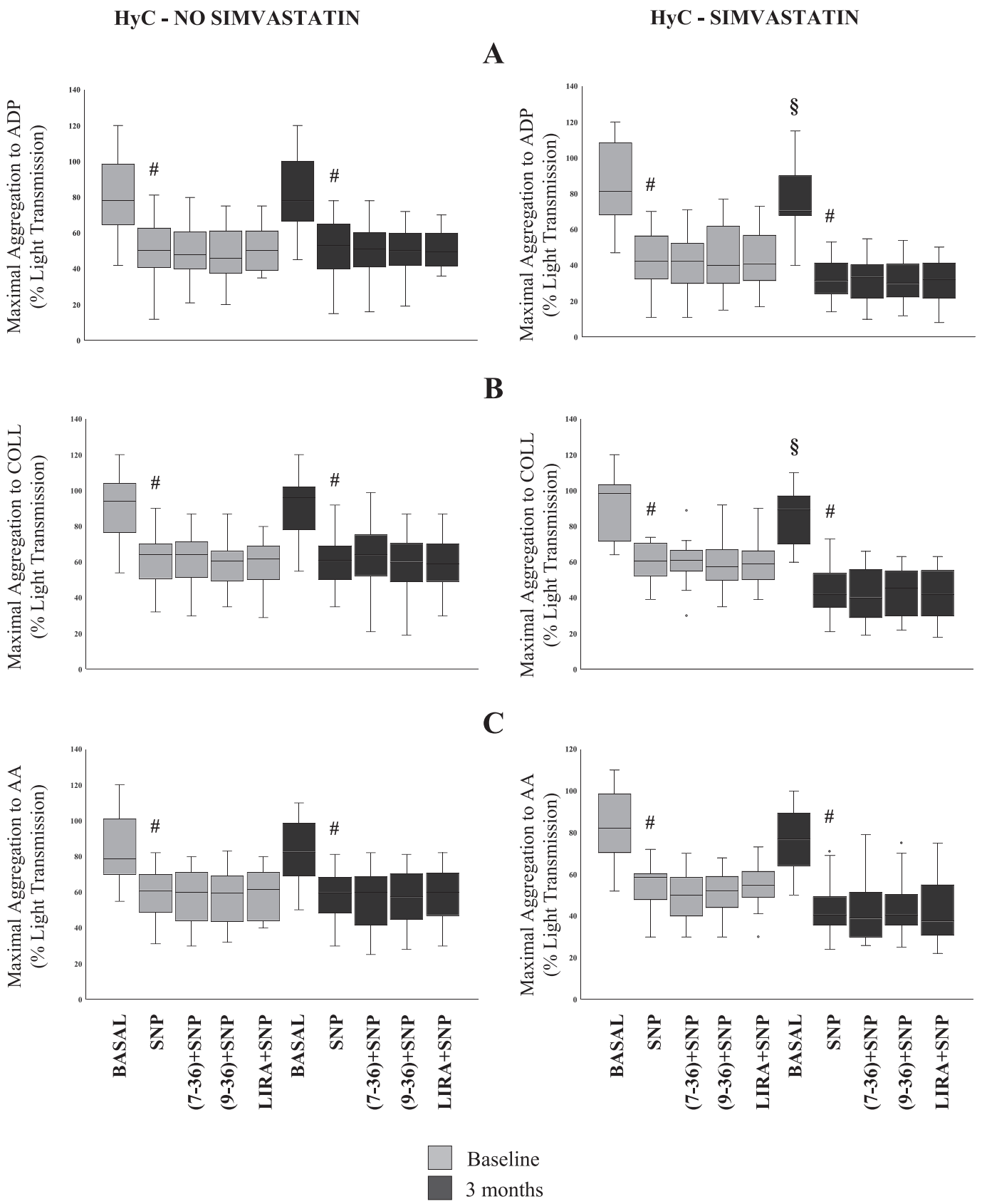
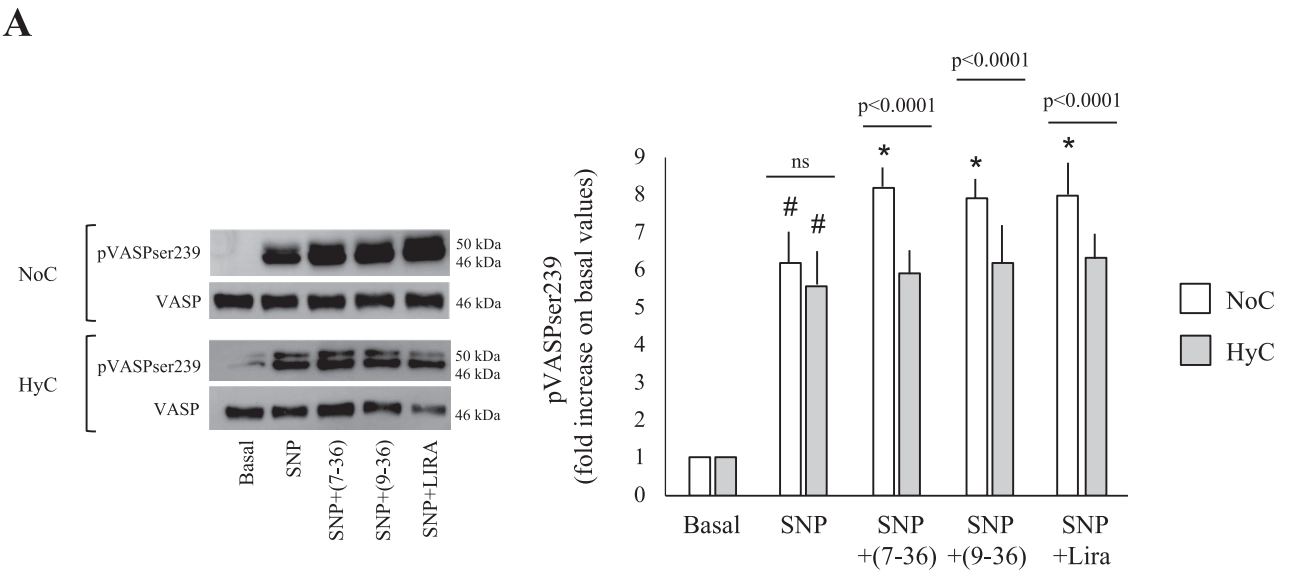
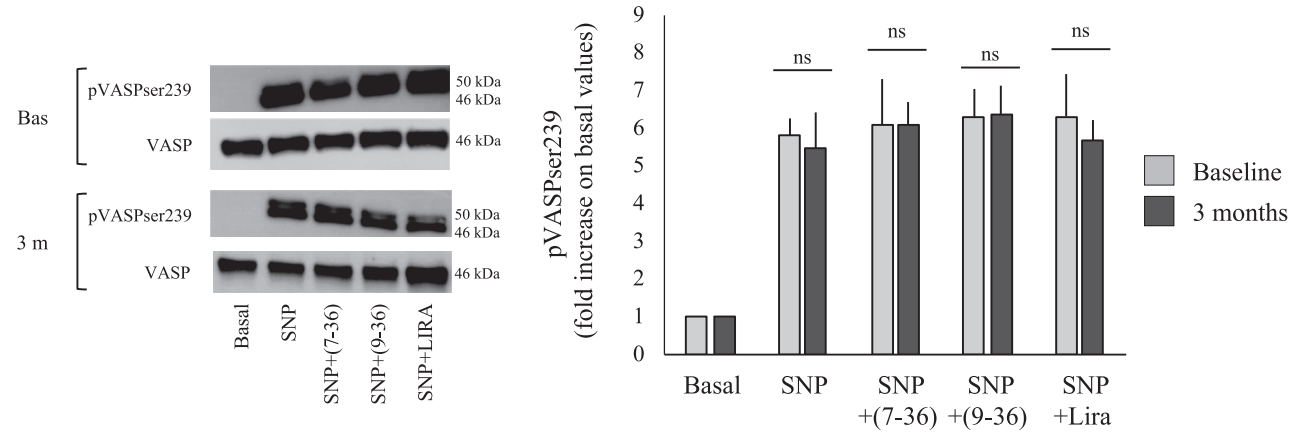


Fig. 2. Effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on platelet aggregation induced by ADP (5 μmol/l) (panel A), collagen (2 mg/l) (panel B) and arachidonic acid (AA) (100 μmol/l) (panel C) in the presence of sodium nitroprusside (SNP) (5 μmol/l) in hypercholesterolemia at baseline and after a-three month treatment with diet alone (on the left) (Hyc–no simvastatin) ($n = 26$) or diet plus 40 mg/die simvastatin (on the right) (Hyc–simvastatin) ($n = 18$). Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range; open circles: outliers. Significance of intra-group: 1-way repeated measures ANOVA ($p < 0.0001$) followed by Bonferroni test: #$p < 0.05$ versus basal. Intragroup difference between baseline and after 3 months was estimated by paired *t*-test: §$p < 0.0.5$ versus basal at baseline.





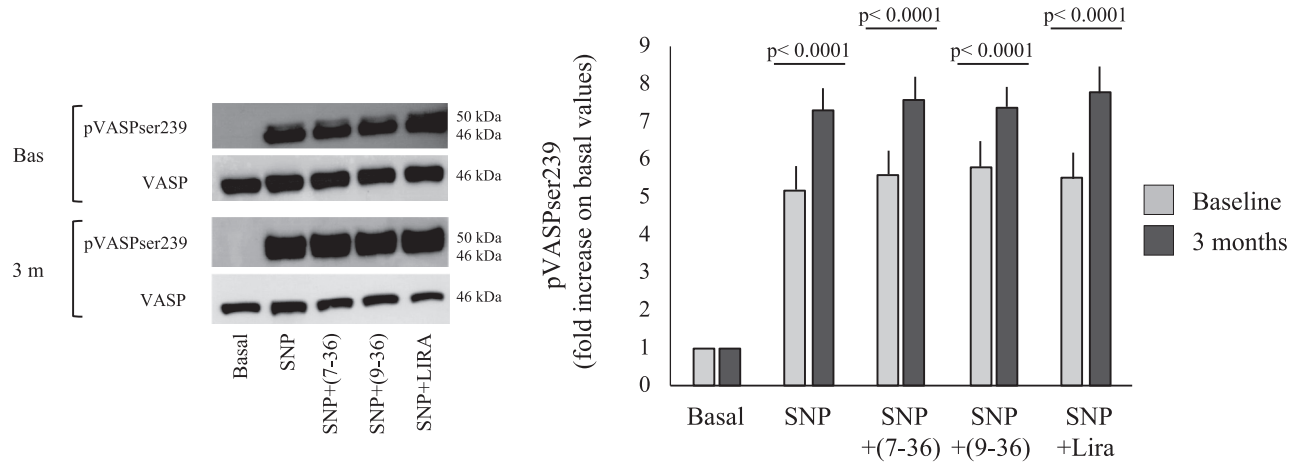


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Fig. 3. Representative blots and densitometric analyses of the effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on the platelet phosphorylation levels of VASP (pVASP) at ser239 induced by sodium nitroprusside (SNP) (5 μmol/l, 5 min) in normocholesterolemia (NoC) ($n = 10$) or hypercholesterolemia (HyC) (n = 10) (Panel A) and in hypercholesterolemia at baseline and after a-three month treatment with diet alone (Hyc–no simvastatin) (n = 10) (Panel B) or diet plus 40 mg/die simvastatin (Hyc–simvastatin) (n = 10) (Panel C). Significance of intra-group: 1-way repeated measures ANOVA ($p < 0.0001$) followed by Bonferroni test: #p < 0.05 versus respective basal, *p < 0.05 vs SNP alone. Significance between NoC and HyC was estimated by *t*-test Student. Intragroup difference between baseline and after 3 months was estimated by paired *t*-test.

We found that the SNP-induced pVASP levels: i) increased both in NoC and HyC (vs basal values: $p < 0.0001$ for both) without significant differences between the two groups, ii) rose from 6.2 to 8.2 with GLP-1 (7-36) ($p < 0.05$), to 7.9 with GLP-1 (9-36) (p < 0.05), and to 7.6 with Liraglutide (p < 0.05) in NoC and from 5.6 to 5.9 with GLP-1 (7-36) (p = ns), to 6.2 with GLP-1 (9-36) ($p = 0.01$), and to 6.3 with Liraglutide (p = ns) in HyC (Fig. 3A). Between HyC and NoC no difference was found in terms of expression of total VASP (A.U.) (9122 ± 2022 vs 10,329 ± 2502, respectively, p = ns) and pVASP-ser239 with no stimulus (1527 ± 458 vs 1217 ± 669, respectively, p = ns).

In the no simvastatin therapy group, after 3 months of follow-up no change was observed in platelet response to SNP with or without GLP-1 related peptides (Fig. 3B).

In patients receiving simvastatin, an improvement of platelet sensitivity to NO was observed since the SNP-induced pVASP-ser239 was significantly higher after three months of treatment (versus baseline: $p < 0.0001$) (Fig. 3C). However, in these patients, platelet preincubation with each GLP-1-related peptide did not significantly influence pVASP-ser239 amounts induced by SNP: from 7.3 to 7.6 with GLP-1 (7-36) (p = ns), to 7.6 with GLP-1 (9-36) (p = ns), and to 7.8 with Liraglutide (p = ns).

3.3. GLP-1 effects on Collagen- and AA-induced Akt and ERK-1/2 phosphorylation

WP samples from HyC and NoC were stimulated by collagen or AA both in the absence and presence of GLP-1 related peptides and pAkt and pERK-1/2 levels evaluated by western blot.

In response to collagen, pAkt and pERK-2 levels: i) increased both in NoC and HyC (vs basal values: $p < 0.0001$ for both) with higher values in HyC than in NoC for pAkt ($p = 0.004$) and pERK-2 ($p = 0.03$), ii) decreased after platelet preincubation with each GLP-1 peptide in NoC ($p < 0.05$) but not in HyC (versus collagen alone: p = ns) (Fig. 4A).

In response to AA, pAkt and pERK-2 levels: i) increased both in NoC and HyC (vs basal values: $p < 0.0001$ for both) with a trend of higher values in HyC than in NoC for both pAkt ($p = 0.063$) (Fig. 4A) and pERK-2 ($p = 0.058$), ii) decreased after platelet preincubation with each GLP-1 peptide in NoC (p < 0.05 for both), but not in HyC (versus AA alone: p = ns) (Fig. 4B).

Patients receiving simvastatin showed decreased platelet pAkt and pErk-2 amounts induced by collagen ($p < 0.0001$ for both) (Fig. 5A) or AA (Fig. 5B) (p = 0.004 and $p < 0.0001$, respectively). However, platelet preincubation with GLP-1 related peptides did not significantly modify pAkt and pErk-2 levels induced by collagen or AA (versus collagen or AA alone: p = ns for each GLP-1 peptide). In the no simvastatin therapy subjects, no change was observed in platelet response to collagen or AA with or without GLP-1 related peptides (data not shown).

3.4. GLP-1 influence on ROS production

WP samples from HyC and NoC were stimulated by AA both in the absence and presence of GLP-1 related peptides and ROS production was evaluated by DCF-DA assay.

HyC, if compared to NoC subjects, showed higher platelet ROS levels at basal (p < 0.0001) and after AA stimulation (p < 0.0001). The AA-induced ROS levels: i) were reduced by 34% with GLP-1(7-36) ($p < 0.005$), 32% with GLP-1 (9-36) ($p < 0.001$) and 33% with Liraglutide (p < 0.0001) in NoC, ii) were not influenced by GLP-1 peptides in HyC (versus AA alone: p = ns for each GLP-1 peptide) (Fig. 6A).

In the no simvastatin therapy subjects, no change was observed in platelet ROS at basal and after AA stimulation with or without GLP-1 related peptides (Fig. 6B).

In patients receiving simvastatin, compared with baseline values, platelets showed a reduction of ROS levels without ($p = 0.003$) and with AA stimulation ($p = 0.001$) (Fig. 6C). However, the preincubation with GLP-1 peptide did not interfere with the AA-induced ROS generation (DCF fluorescence): from 3592 ± 656 to 3059 ± 586 with GLP-1 (7-36) (ns), to 2861 ± 493 with GLP-1 (9-36) (ns), to 3251 ± 747 with Liraglutide (ns).

4. Discussion

Main findings of this study were that in patients affected by primary HyC: ii) GLP-1 effects on platelets were deeply impaired, ii) a lipid-lowering treatment with simvastatin for three months reduced platelet aggregation/activation, improved the inhibitory action of NO but did not restore platelet sensitivity to GLP-1. The observation of altered GLP-1 action was found for the native form GLP-1 (7-36), the truncated peptide GLP-1 (9-36) and the analogue Liraglutide.

GLP-1 is the object of investigation for not only its contribution to glucose homeostasis but also for a variety of biological actions beyond its metabolic effects [1,22] mediated by both GLP-1R dependent and independent pathways [23].

Among them, GLP-1 modulates some aspects of platelet function with potential protective roles on the cardiovascular system, thus suggesting that a reduced and/or impaired action of GLP-1 on platelets could be involved in the platelet hyperreactivity described in metabolic disorders such as diabetes [24,25] and dyslipidemia [22,23,26,27].

Actually, in the present study platelets from HyC patients showed enhanced reactivity as mirrored by: i) higher aggregability to ADP, collagen, AA, ii) higher ROS production, iii) reduced sensitivity to NO pathway and iv) increased activation of PI3K/Akt and MAPK/ERK-2 pathways. In such scenario, platelet exposure to GLP-1 related peptides did not modify the antiaggregating effects of exogenous NO. Furthermore, we also found a resistance to GLP-1 ability to reduce ROS generation and activation of both PI3K/Akt and MAPK/ERK-2 pathways when platelets were stimulated by collagen or AA. MAPK/ERK-1/2 and PI3K/Akt regulate platelet functions [28,29] and PKG activation is involved in their modulation [8,30]. Since ROS affect platelet function by reacting with NO and attenuating NO bioavailability [31], present findings suggest that the inability of GLP-1 to reduce ROS production may explain why the preincubation with GLP-1 (7-36), GLP-1 (9-36) or Liraglutide failed to increase the antiaggregating effects of NO, the phosphorylation at ser-239 of the PKG activation marker VASP and to reduce the AA- and collagen-induced pAkt and pERK-2 levels. The exact mechanisms of platelet resistance to the GLP-1 effects are unclear, however it is unlikely that GLP-1R is involved because GLP-1 effects on platelets occur independently of GLP-1R [8].

An impaired action of GLP-1 has been described in type 2 diabetes [32] justifying GLP-1-based therapies as established treatment options for the metabolic control of the disease [33]. Uncertainty remains about the cardiovascular effects of GLP-1R agonists. The effect of these treatments on individual cardiovascular outcomes varied between drugs effective in reducing cardiovascular events, such as Liraglutide

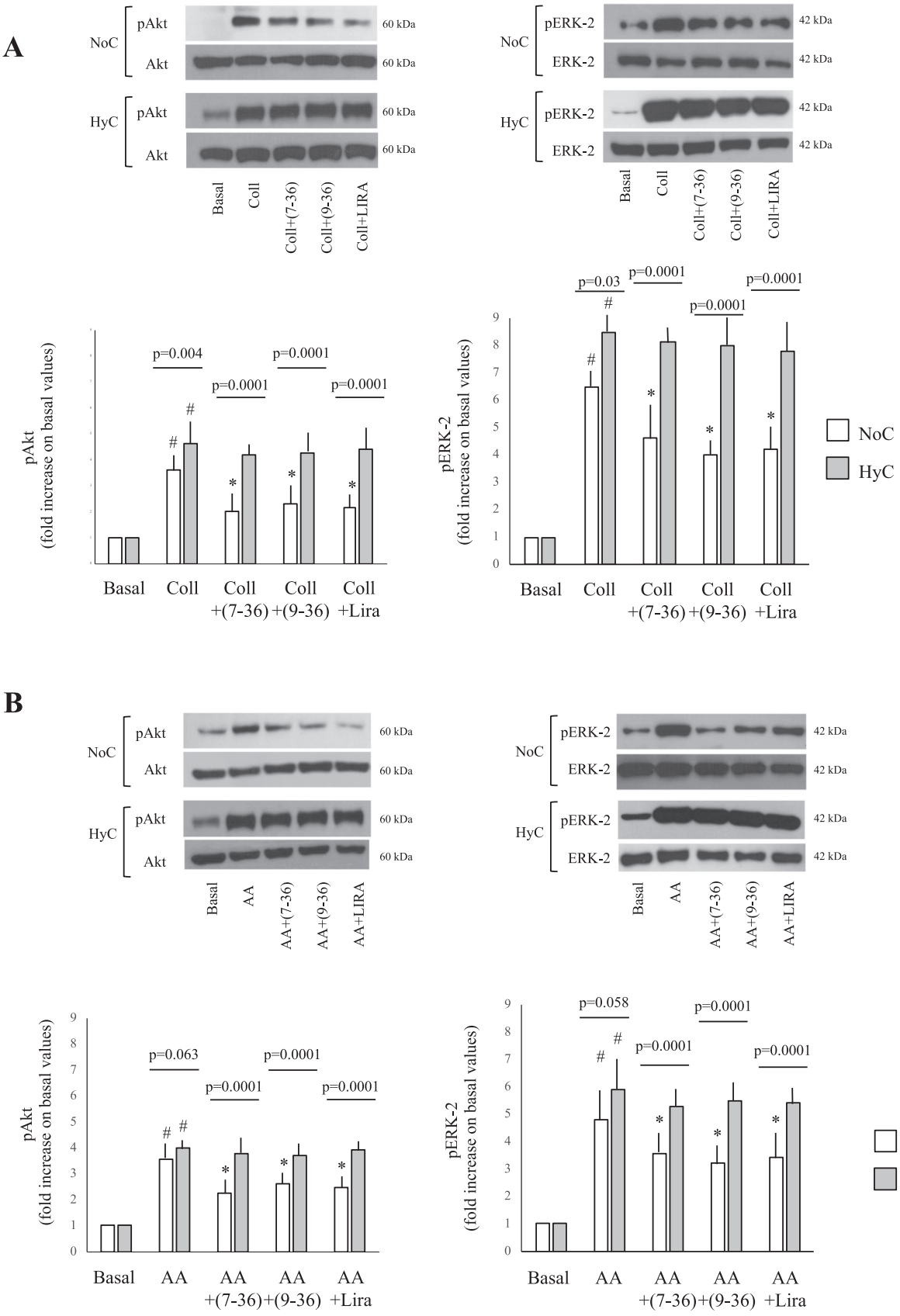

Fig. 4. Representative blots and densitometric analyses of the effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on the platelet phosphorylation levels of Akt (pAkt) or ERK-2 (pERK-2) stimulated by collagen (2 mg/l) (Panel A) or arachidonic acid (AA) (100 μmol/l) (Panel B) in normocholesterolemia (NoC) (n = 10) or hypercholesterolemia (HyC) (n = 10). Significance of intra-group: 1-way repeated measures ANOVA followed by Bonferroni test: #p < 0.05 versus respective basal, *p < 0.05 vs collagen or AA alone. Significance between NoC and HyC was estimated by *t*-test Student.

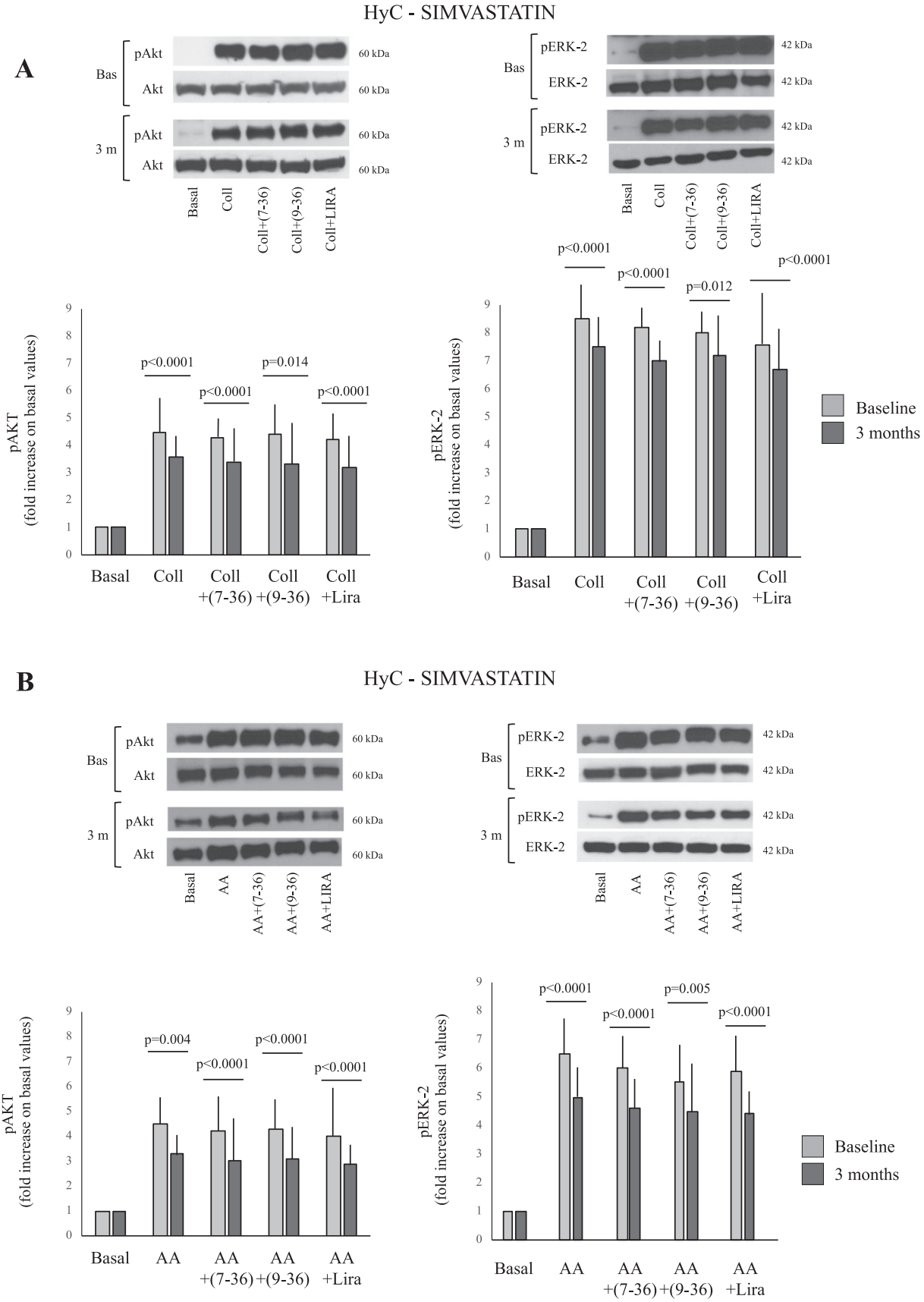

Fig. 5. Representative blots and densitometric analyses of the effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on the platelet phosphorylation levels of Akt (pAkt) or ERK-2 (pERK-2) stimulated by collagen (2 mg/l) (Panel A) or arachidonic acid (AA) (100 μmol/l) (Panel B) in hypercholesterolemics at baseline and after a-three month treatment with diet plus 40 mg/die simvastatin (Hyc–simvastatin) (n = 10). Intragroup difference between baseline and after 3 months was estimated by paired *t*-test.

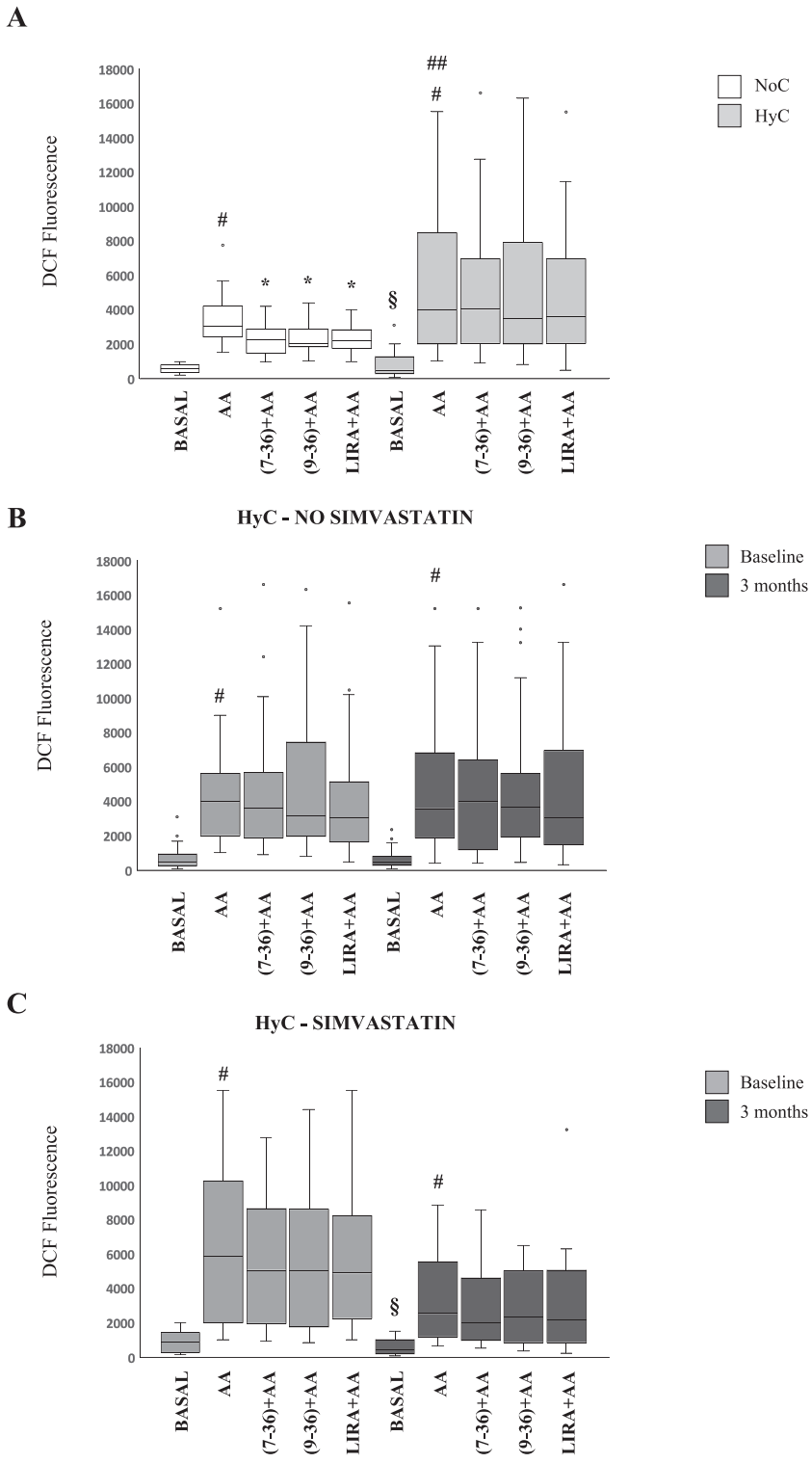

Fig. 6. Effects of a 15-min preincubation with GLP-1 (7-36), GLP-1 (9-36) and Liraglutide (100 nmol/l) on the platelet generation of reactive oxygen species stimulated by arachidonic acid (AA) (100 μmol/l) in normocholesterolemia (NoC) (n = 12) or hypercholesterolemia (HyC) (n = 12) (Panel A) and in hypercholesterolemia at baseline and after a-three month treatment with diet alone (Panel B) (n = 12) or diet plus 40 mg/die simvastatin (Panel C) (n = 12). Solid lines: median values; boxes: interquartile range; whiskers: nonoutlier range; open circles: outliers. Significance of intragroup: 1-way repeated measures ANOVA ($p < 0.0001$) followed by Bonferroni test: #$p < 0.05$ versus respective basal, *$p < 0.05$ vs AA alone. Significance between NoC and HyC was estimated by t-test Student: §$p = 0.002$ versus basal NoC, ##$p < 0.0001$ vs AA NoC. Intragroup difference between baseline and after 3 months was estimated by paired t-test: §$p < 0.0001$ versus basal at baseline.

and Semaglutide showing structural homology to native GLP-1 [3–6] and other GLP-1R agonists, such as exendin-4 based drugs Lixisenatide and Exenatide, whose cardiovascular benefits were inconsistent [3–5]. Since dyslipidemia is present in a large number of patients with diabetes, we cannot exclude that, at least partially, the presence of dyslipidemia could negatively influence per se GLP-1 effects on cardiovascular system.

The causes of the enhanced platelet hyperaggregability and the defective GLP-1 actions in dyslipidemia can be multifactorial, however the strong correlation with LDL cholesterol underlines the role of cholesterol as major determinant of platelet hyperreactivity with a putative role also in the impaired response to GLP-1.

Actually, the ratio of membrane cholesterol/phospholipids increases in HyC [34] and it has been associated with platelet hyperreactivity [35,36] supporting the benefit of antiplatelet drugs to treat and prevent cardiovascular events.

Statins decrease cardiovascular events by LDL cholesterol reduction and/or anti-atherothrombotic effects including an influence on platelet

function in HyC [14]. For this reason, we wondered whether a lipid-lowering therapy with statin was also able to restore platelet sensitivity to the effects of GLP-1 related peptides. Indeed, we found that a three-month treatment with simvastatin lowered plasma total and LDL cholesterol, reduced platelet aggregation to ADP, collagen and AA, ROS production and improved the inhibitory action of NO, thus confirming the benefit of this drug on platelet function. However, the treatment was ineffective in correcting GLP1-1 effects on platelets. In particular, the ability of GLP-1 to increase NO effects on aggregation and PKG activation, to reduce the agonist-induced activation of PI3K/Akt and MAPK/ERK-2 pathways, and to reduce ROS levels were lost before and after simvastatin therapy. These defective GLP-1 actions were found for GLP-1 (7-36), GLP-1 (9-36) and Liraglutide. A possible explanation of platelet insensitivity to GLP-1 after simvastatin therapy could imply that target site of GLP-1 and simvastatin is shared. Actually, we should consider that in ROS attenuation by GLP-1 in NoC an inhibitory GLP-1 effect on the modulator of intracellular ROS generation NADPH oxidase is involved [8]. Since also the antiplatelet activity of statins seems to be dependent, at least in part, upon the inhibition of platelet NADPH oxidase-derived ROS formation [37], one can suppose that platelet exposure to GLP-1 after a treatment with a stronger inhibitor of platelet NADPH-oxidase was no longer able to further modify enzymatic pathways involved in ROS generation and increase NO bioavailability.

On the other hand, differently from statins, GLP-1 alone in vitro does not interfere on platelet aggregation induced by agonists thus confirming its low ability to interfere with pathways directly involved in platelet inhibition. The absence of an additive effect between GLP-1 and simvastatin on platelets would support this hypothesis even if other studies are needed to shed more light on this aspect.

Of course, we cannot exclude that a different clinical approach (longer follow-up, hydrophilic instead of lipophilic statin, other simvastatin dosage) would have led to different results.

In conclusion, the present study, for the first time, demonstrated that HyC is characterized by a resistance to GLP-1 related peptides effects on platelets and this impairment is not corrected by a short-time treatment with simvastatin.

Declaration of Competing Interest

None.

Acknowledgments

We would like to thank the Società Italiana per lo Studio dell'Aterosclerosi (SISA) for a fellowship granted to CB. This study was partially funded by a grant from the Department of Clinical and Biological Sciences of Turin University (Ricerca Locale Ex-60%) to I.R. (RUSI_RILO_15).

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