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Hypercoagulable state in hypercholesterolemic subjects assessed by platelet-dependent thrombin generation
In vitro effect of cerivastatin

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Abstract. – ***Background:*** **Hypercholesterolemia is an important risk factor to develop acute thrombotic complications of atherosclerosis like to myocardial infarction and ischemic stroke. Platelets and coagulation factors are strictly involved in the genesis of such thrombotic events and their hyperactivity in hypercholesterolemic patients has been previously reported. Moreover some cholesterol-lowering molecules (statins) seem to be able of reducing platelet activity.**

Methods: **We performed platelet-dependent thrombin generation (colorimetric method) to assess the coagulative potential of 40 caucasian hypercholesterolemic subjects with respect to normal controls and to the grade of hypercholesterolemia. Moreover we observed the effect of platelets from hypercholesterolemics on thrombin generation in plasma from normal subjects. The effect of Cerivastatin on thrombin generation was evaluated too.**

Results: **Our data show an increased thrombin generation both in mild and high hypercholesterolemic subjects with respect to controls (424.6±30.5 vs 197.1±27.4 mIU/ml). No significant difference in the amount of thrombin generation was found between mild and high hypercholesterolemics (399.6±20.7 vs 440.2±21.4 mIU/ml). Platelets directly influence thrombin generation and they present an intrinsic hyperactivity that can be modulated by Cerivastatin (223.6±24.8 vs 424.6±30.5 mIU/ml).**

Conclusions: **Mild hypercholesterolemia is associated with an increased thrombinic potential that may be considered an added risk factor to develop thrombotic events. Platelets directly influences this hypercogulative state and Cerivastatin is able to reduce thrombin generation by way of a direct interaction with platelets.**

Key Words:

Dyslipidaemia, Platelets, Coagulation, Statins.

Introduction

Hypercholesterolemia is an important risk factor for atherosclerotic disease and is often associated with acute events like to myocardial infarction and cerebrovascular disorders[1-3]. The endothelial damage at the plaque level induces a synergism between platelets and the coagulation system which is the leading event to develop the thrombotic or thromboembolic state liable for such ischemic events. It is now known how important is the role of activated platelets in the growth of the "white thrombus" and in the activity of the prothrombinase complex to produce thrombin which is the essential step either in physiologic coagulation or pathologic thrombosis[4-11].

Some studies have demonstrated increased platelet and coagulation activity in hypercholesterolemic patients without examining their interactions[12-32] and a reduced platelet activity has been described after treatment with agents reducing total blood cholesterol levels[24,25,28,33-35]. In 1992 Aronson and others[36] developed an experimental method for measuring the platelet-dependent thrombin generation to assess the interactions between platelet activity and the coagulation system. In a study by Aoki and others[37] the platelet dependent thrombin generation was evaluated in Japanese hyperlipidemic subjects showing an increased thrombin generation. In 1996 Szczeklik and others performed an indirect observation of platelet hyperactivity in hypercholesterolemic subjects showing a blunted inhibition of thrombin generation by

aspirin[38]. The aim of our study was to evaluate the amount of platelet-dependent thrombin generation in a caucasian hypercholesterolemic group with respect to normolipidemics and in relation to serum total cholesterol (T-Chol) and serum HDL cholesterol (HDL-Chol) level. The cut-off was at 250 mg/dl and 40 mg/dl respectively[39].

Moreover we performed an analysis of thrombin generation on platelet poor plasma (PPP) from normal subjects in the presence of washed platelets from hypercholesterolemic subjects and in the reverse condition in order to detect the role of platelets in the extent of thrombin generation (mixed experiment).

The effect of Cerivastatin (in vitro) on platelet-dependent thrombin generation was evaluated too.

Materials and Methods

Subjects

We studied 40 patients with hypercholesterolemia (23 male, 17 female, mean age 54.7±10, T-Chol level 279.6±35.7 mg/dl, HDL Chol level 30.1±7.6, triglicerides 108.9±12.8 mg/dl) and 40 normal subjects (21 male, 19 female, mean age 43.1±14.6, T-Chol level 171.2±15 mg/dl, HDL-Chol level 42.4±4.2 mg/dl, triglicerides 96.7±14.5 mg/dl) as the control group.

All subjects had no previous cardiac or cerebral acute ischemic events, none was affected by hypertension, diabetes mellitus or other underlying disease, none had a personal or a familial history of venous thrombosis or bleeding tendency. Renal and hepatic function were normal, none was a smoker and no subject received lipid-lowering and antiplatelet or anticoagulant agents. All subjects gave their informed consent.

Blood collection

Venous blood was collected at morning from resting and fasting subjects without stasis by a 19 gauge needle and was stored in a plastic syringe (3.8 sodium citrate 1:9 blood) to detect platelet-dependent thrombin generation; another part was collected into a plastic tube to obtain serum for developing cholesterol measurements.

Platelet-dependent thrombin generation

The thrombin generation was measured according to the method of Aronson and others[36] modified by Aoki and others[37] with slight modifications. In brief, the collected venous blood was centrifuged at 100 g for 20 min at 20° C to obtain platelet-rich plasma (PRP). This was separated from the top two-thirds of the supernatant to avoid contamination by other cells. The remaining blood was centrifuged at 1500 g for 10 min to obtain platelet-poor plasma (PPP). The platelet count in the PRP was performed by a Coulter Counter (Coulter Electronics Ltd., UK) and the platelet concentration was adjusted to 300×10^9/liter with PPP. 0.5 ml of PRP were placed into polypropilene tubes and 20 µl of 1 mol/liter calcium chloride was added to initiate clotting. Samples (10 µl) were added to the wells of a microtiter plate containing 90 µl of 3.8 sodium citrate at 5 min intervals for 30 min. Color was developed for 2 min by the addition of 50 µl of 0.5 mmol/liter S-2238 (Chromogenix, Sweden), a thrombin-specific substrate, in 1 mol/liter Tris (pH 8.1). The absorbance of the released color product was measured at a wavelength of 405-nm using a microtiter plate reader (Bio-Rad, model 550); measurements were obtained in duplicate at each time point and the amount of thrombin generation was calculated from a standard curve.

Cholesterol and triglycerides measurements

Serum T-Chol and HDL-Chol were measured by available commarcial kits on a reflotron® analyzer (Boehringer Mannheim).

Washed platelets

After PRP was obtained, washed platelets were isolated by a multiple centrifugation technique in Tyrode's Buffer (137 mM NaCl, 2.8 mM KCl, 1 nM $MgCl_2$, 12 mM $NaHCO_3$, 0.4 mM Na_2HPO_4, 0.35% serum albumin, 10 mM HEPES, 5.5 mM dextrose pH 7.4) according to Mustard[40].

Mixed experiment

Washed platelets from hypercholesterolemic subjects were added to PPP from normal subjects at a final concentration of 300×10^9/L and washed platelets from normal subjects to PPP from from hypercholes-

terolemics at the same final concentrations. Platelet-dependent thrombin generation was evaluated as above described.

Pharmacological study

In all subjects platelet-dependent thrombin generation was evaluated after incubation with cerivastatin at final concentration of 2 µg/L for 1 hour. The dose of Ceivastatin was applied in relation to the plasmatic level obtained after oral administration of the drug[41].

Statistical analysis

Results are shown as the mean value ± standard error (SEM). We performed the statystical analysis on all the hypercholesterolemic subjects (Group A) and in two subgroups of patients (cut off level of T-Chol at 250 mg/dl, Group B < 250 mg/dl, n = 18, Group C > 250 mg/dl, n = 22) with respect to controls (Group D). We studied our population with respect to HDL-Chol levels too. The cut off was at 40 mg/dl (Group E > 40 mg/dl, n = 34, Group F < 40 mg/dl, n = 46, 6 subjects had normal T-Chol and low HDL-Chol). Moreover we performed an analysis on the results from mixed experiment (n = 20 PPP from normal plus platelets from hypercholesterolemic subjects and n = 20 in the reverse condition). All data were analyzed by ANOVA and the Bonferroni/Dann method was used for multiple comparisons when a significant difference was observed. A p value < 0.01 was accepted as indicating a statistical significance. The calculations were performed using Statwiew (Abacus Concepts Inc).

Results

Platelet-dependent thrombin generation was significantly increased in hypercholesterolemic patients (Group A) with respect to controls (Group D) (424.6±30.5 mIU/ml vs 197.1±27.4 mIU/ml) ($p < 0.001$) (Figure 1). A significant increase in thrombin generation was also detected in Group B and C (B= 399.6±20.7 mIU/ml, C = 440.2±21.4 mIU/ml) ($p < 0.001$) while no statistical significance was found by the comparison of group B and C ($p = 0.997$) (Figure 2). There was no significant difference of thrombin generation between men and women in hypercholesterolemic (418.5±27.6 mIU/ml vs 430.8 ± 33.8 mIU/ml) and in control subjects (199.9 ± 30.8 mIU/ml vs 196.3±24.3 mIU/ml). The analysis of Group E and F showed a significantly enhanced thrombin generation in subjects with lower HDL-Chol levels (data not shown).

In the presence of platelets from hypercholesterolemic subjects PPP of normals showed an enhanced thrombin generation with respect to the autologus test (379.6 ± 28.4

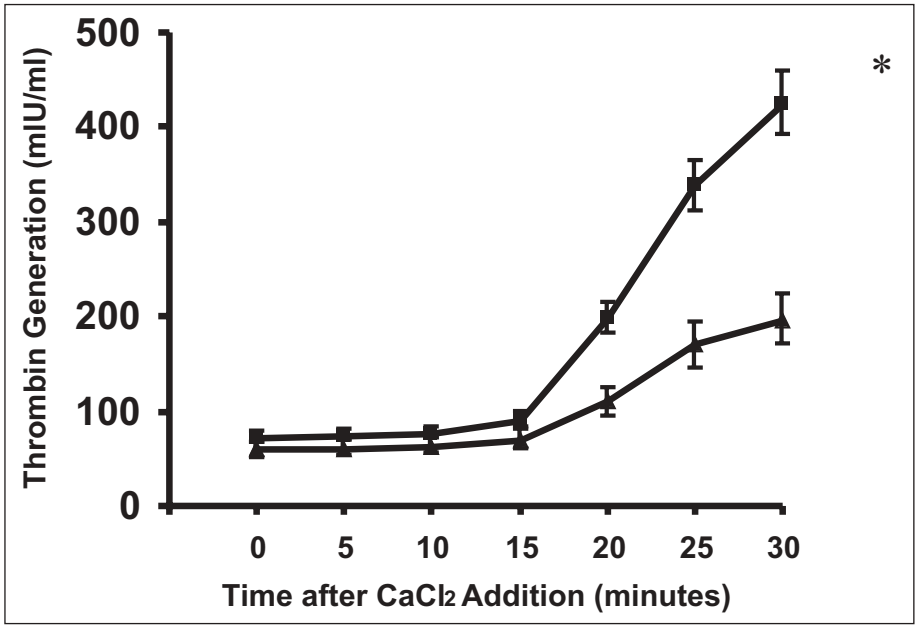

Figure 1. Platelet-dependent thrombin generation in hypercholesterolemic (square) (n = 40) and in control subjects (triangles) (n = 40). *p < 0.001. data are shown as mean value ± SEM.

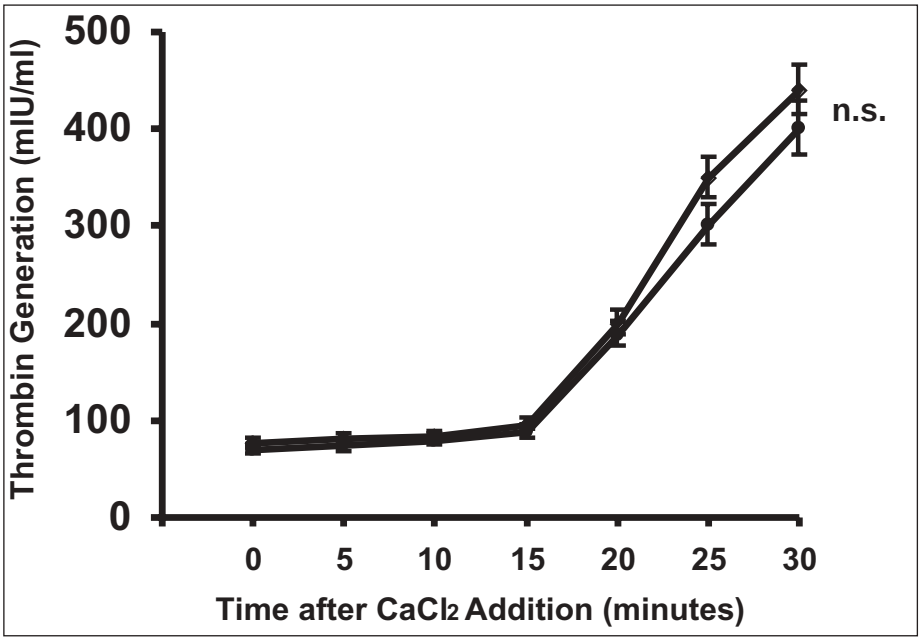

Figure 2. Platelet-dependent thrombin generation in hypercholesterolemic < 250 mg/dl (circles) (n = 18) and > 250 mg/dl (diamonds) (n = 22). n.s. = not significant, data are shown as mean value ± SEM.

mIU/ml vs 197.1±27.4 mIU/ml) (p< 0.001) (Figure 3). When platelets from normal subjects were added to PPP from hypercholesterolemics the amount of thrombin generation was significantly reduced with respect to the autologus test (258.6±22.8 mIU/ml vs 418.5±27.6 mIU/ml) (p< 0.001) (Figure 4). After addition of Cerivastatin, thrombin generation was significantly reduced with respect to the basal values either in hypercholesterolemic (223.6±24.8 mIU/ml vs 424.6± 30.5 mIU/ml) (p < 0.001) (Figure 5) or normal subjects (108.9±19.6 mIU/ml vs 197.1±27.4 mIU/ml) (p < 0.01) (Figure 6).

When Tyrode's buffer alone was added to PPP no difference in thrombin generation was observed with respect too baseline test (data not shown).

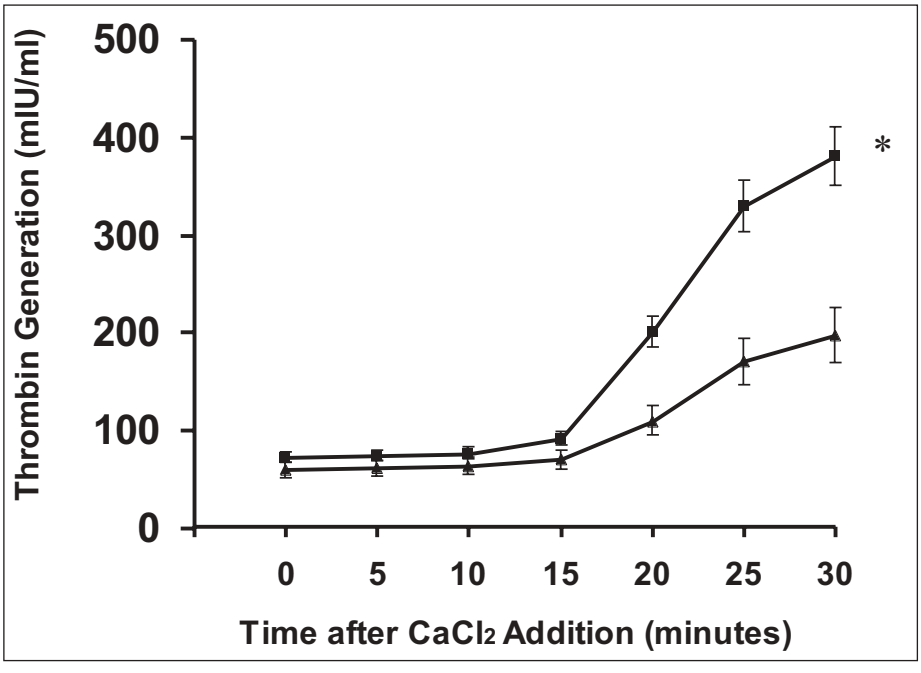

Figure 3. Platelet-dependent thrombin generation in PPP from normal subjects with platelets from hypercholesterolemics (square) and in the autologus test (triangles) (n = 40). * p < 0.001. data are shown as mean value ± SEM.

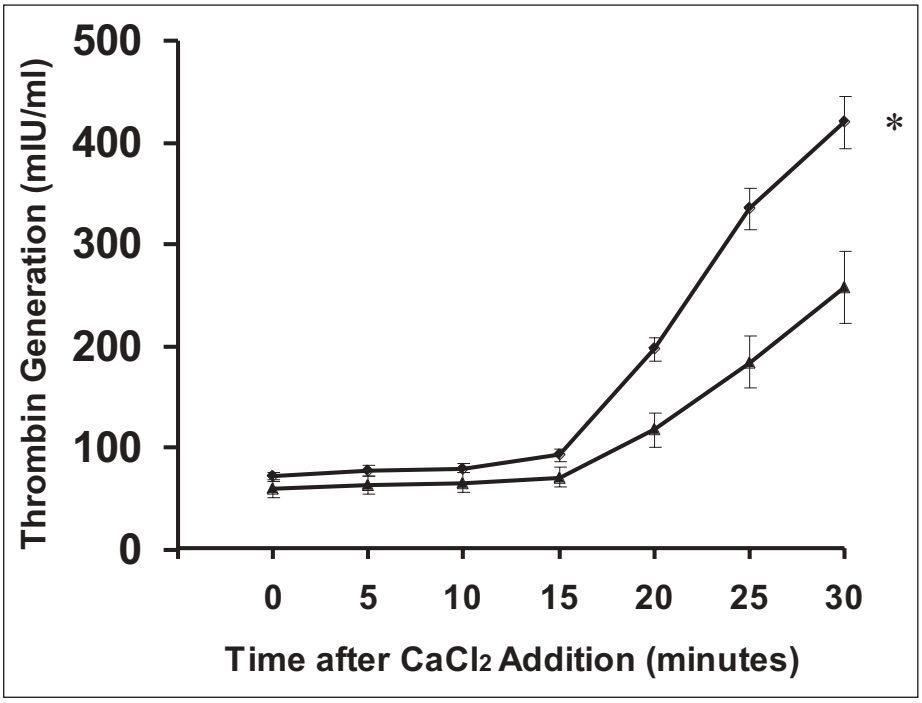

Figure 4. Platelet-dependent thrombin generation in PPP from hypercholesterolemics with platelets from normals (triangles) and in the autologus test (square) (n = 40). * p< 0.001, data are shown as mean value ± SEM.

Discussion

In our study platelets strongly influences thrombin generation which is enhanced in hypercholesterolemic subjects with respect to normal controls. Some studies have confirmed an hypercoagulable state in hypercholesterolemic subjects[25-32] while others show conflicting results[42,43]. The relation between hypercholesterolemia and increased platelet reactivity is still not completely understood, alterations in HDL-Chol or LDL-Chol subfractions has been associated with reduced or enhanced platelet activity but

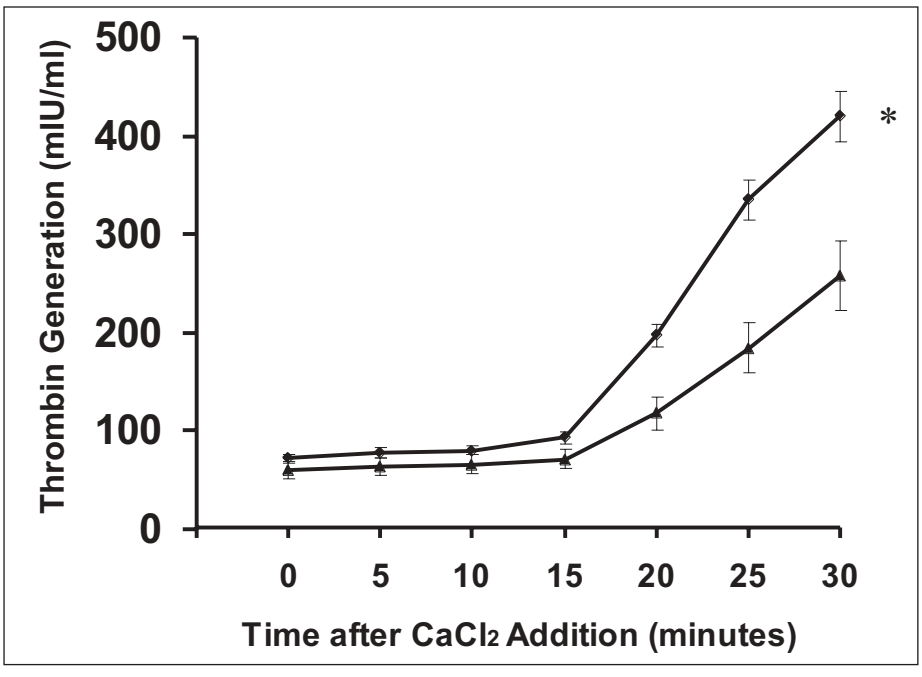

Figure 5. Hypercholesterolemic subjects. Platelet-dependent thrombin generation in before (square) (n = 40) and after (triangles) (n = 40) Cerivastatin addition (2 µg/ml). * p < 0.001. data are shown as mean value ± SEM.

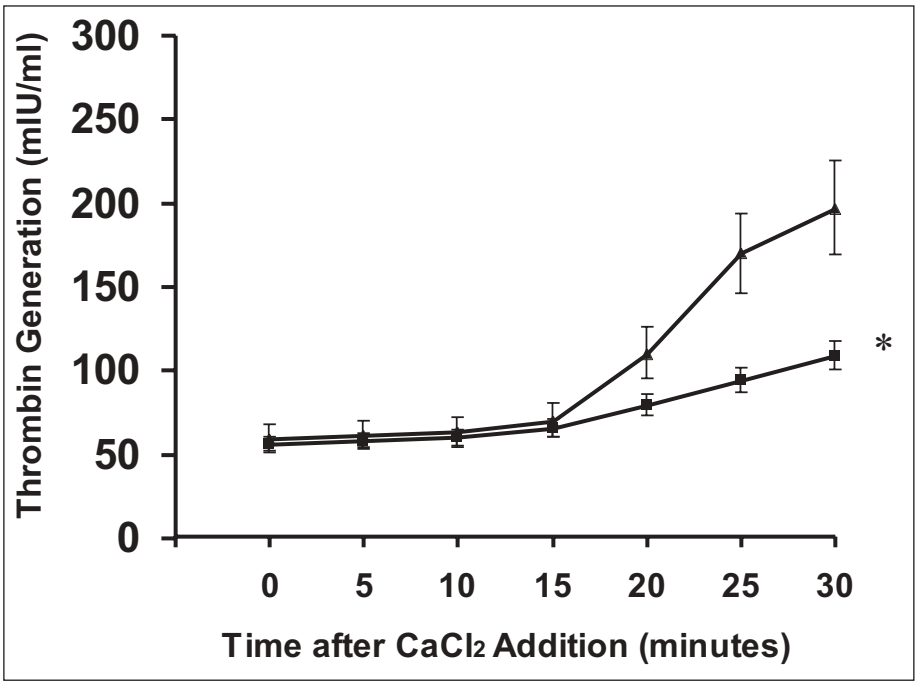

Figure 6. Normal subjects. Platelet-dependent thrombin generation. before (triangles) (n = 40) and after (square) (n = 40) Cerivastatin addition (2 µg/ml). *p < 0.001. data are shown as mean value ± SEM.

further studies are needed to evaluate this possibility[28,30].

Our data show no direct correlation between the degree of hypercholesterolemia and thrombin generation. In fact subjects with the highest T-Chol level have a similar amount of thrombin generation with respect to the group with mild cholesterol increase. Low HDL-Chol patients had an higher thrombin generation but most of them had elevated level of T-Chol too. Only six subjects had low HDL-Chol associated with normal T-Chol and this group had no statistic power to be analyzed. However the single data show that these subjects had an amount of thrombin generation similar to normolypidemic controls. The results from the mixed experiment show that the amount of thrombin generation is directly influenced by platelets. Cells from hypercholesterolemic subjects are able of enhancing thrombin generation in plasma from normal subjects and in the reverse condition thrombin generation is reduced with respect to the autologus test in hypercholesterolemic subjects. These data indicate that platelet hyperactivity in hypercholesterolemic subjects is not due to a circulating factor but to a modification in platelets themselves. Moreover in the presence of Cerivastatin the platelet-dependent thrombin generation was significantly reduced either in normal or hypercholesterolemic subjects suggesting a direct action of the drug on platelet activity indipendently from the cholesterol lowering action. Finally in our study sex does not influence thrombin generation either in hypercholesterolemic or normal subjects.

Thus the enhanced platelet-dependent thrombin generation may be considered an adjunctive element for the predisposition to develop thrombotic events in patients with mild hypercholesterolemia and Cerivastatin is able of counteracting, in vitro, this mechanism.

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