

Protein S on the surface of plasma lipoproteins: a potential mechanism for protein S delivery to the atherosclerotic plaques?

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Key words: Protein S, Atherosclerotic plaques, Lipoproteins, Phospholipids.

Acknowledgments: The study has been carried out at the Department of Life Sciences and Biotechnology, University of Ferrara, Ferrara, Italy. The study was financially supported by Grant from Italian Ministry of University and Research, University of Ferrara, Italy (FAR 2020, FAR 2021), 5x1000 University of Ferrara grant (2021_NAZ.A_B) and by “Bando FIRD 2022 – BARONI” University of Ferrara grant.

Contributions: AP, CM and MG characterized the patient material and clinical data. BM and FF were responsible for immunodepletion, ELISA and Western blotting analysis. ZN did the statistical analyses and together with BD, MN and MG contributed to the interpretation of the data and drafting of the manuscript. BM and BF designed the study, interpreted the data and wrote the manuscript.

Conflict of interest: The Authors declare no potential conflict of interest.

Funding: None.

Ethical approval: This study was approved by local Ethical Committee (University-Hospital of Ferrara) with protocol number 7-2010. All the recruited subjects signed an informed consent.

Received for publication: 20 June 2022.
Accepted for publication: 15 September 2022.

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Bleeding, Thrombosis and Vascular Biology 2022; 1:45
doi:10.4081/btvb.2022.45

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ABSTRACT

The anticoagulant protein S (PS) binds phospholipids with very high affinity, but PS interaction with lipoproteins and lipid-rich atherosclerotic plaques remains still poorly defined. We investigated PS in plasma lipoproteins and in atherosclerotic plaques from ten patients undergoing endarterectomy. PS was detected by Western blotting after exposure of the necrotic core to liposomes and was found to maintain its ability to bind phosphatidylserine micelles. The amounts of PS bound to low/very low-density lipoproteins in patient’s plasmas were higher and more variable than those detected in healthy subjects. A direct correlation between bound PS and low-density lipoproteins (LDL), plasma levels was found only in patients ($r=0.921$, $p<0.001$), thereby leading to hypothesize that the PS-phospholipids binding may increase by oxidative processes of LDL in atherosclerotic patients. The presence of the PS into the necrotic core of atherosclerotic plaques and on the surface of lipoproteins, particularly the atherogenic LDL, suggests a LDL-based delivery of PS to the atherosclerotic plaques and emphasizes the deep link between plasma lipids and coagulation in cardiovascular diseases.

INTRODUCTION

Atherosclerosis is a lipoprotein-driven disease that leads to plaque formation at specific sites of the arterial vessels through intima inflammation, necrosis, fibrosis, and calcification.¹ Atherosclerosis is also a chronic low-grade inflammatory disease associated with increased expression of the acute phase reactants, like the isoforms of Serum Amyloid A (SAA1 and SAA2) which circulate linked with the high-density-lipoprotein (HDL) and may represent a plasma biomarker for future cardiovascular events.^{2,3} Atherosclerosis is initiated by the subendothelial retention of apolipoprotein B (apoB)-containing lipoproteins, particularly the low-density lipoproteins (LDL), and continues with their oxidation (oxLDL) and with macrophage uptake of oxLDL.⁴ These processes are followed by macrophage transformation to foam cells that play a critical role in the development of atherosclerosis.

Protein S (PS) is a plasma vitamin K-dependent protein, produced by a variety of cell types (hepatocytes, endothelial cells, megakaryocytes, osteoblasts).⁵ PS acts as an anticoagulant, alone and as cofactor for activated protein C

and tissue factor pathway inhibitor.⁶ Beyond the historically well recognized anticoagulant role, PS may influence other crucial pathways. PS can activate TAM receptors, such as Mer,⁷ thereby favoring leukocyte extravasation, inflammatory activation and adhesion of platelets to endothelial cells. By activation of Mer receptor, PS inhibits macrophage scavenger receptor A-mediated acetylated LDL uptake in macrophages, thus reducing the formation of foam cells.⁸ PS is also associated to damaged endothelial surface, to very low-density lipoprotein (VLDL) rich in triglycerides, and localizes in atherosclerotic coronary lesions by immunohistochemistry and Western blotting experiments.⁹ PS also promote phagocytosis of apoptotic cells¹⁰ and mitosis of vascular smooth muscle cells.¹¹ Taking into account these multi-faceted features of PS and its ability to bind biological membranes, particularly the oxidized phospholipid surfaces,¹² it appears plausible that PS may be present both in the necrotic core of the atherosclerotic plaques and on the surface of lipoproteins, including the atherogenic LDL.

Although the presence of PS in the carotid atherosclerotic plaques has been reported,⁹ several questions remain open, particularly concerning the interaction and binding of PS with lipoproteins. In the present study we investigate these issues in a group of patients who underwent endarterectomy, a surgical procedure which allows to remove the atheromatous plaque material and, thus, to perform specific analyses *ex vivo*.

MATERIALS AND METHODS

Subjects

Ten male patients who underwent thrombo-endarterectomy (TEA-P) for severe arterial occlusion¹³ and 10 healthy subjects (HS) - mean age 71.5±5.9 *vs* 28.8±5.3 years, respectively, $p < 0.0001$ - were studied.

PS and lipoproteins plasma assays

Total plasma PS was measured by ELISA with polyclonal anti-PS antibodies.¹⁴ LDL and HDL levels were evaluated by discontinuous gradient density and flotation ultracentrifugation from fresh plasma samples.¹⁵

Atherosclerotic plaques processing

A total of 10 thrombo-endarterectomy (TEA) specimens, which were used for the different experiments, were obtained at surgery from consecutive patients who underwent conventional TEA. 2 mg of necrotic core from carotid artery plaques was used for protein extraction. The samples were homogenized, under cryogenic conditions, in a mortar pestle and proteins extracted using a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1% SDS, 0.1% NP-40, 1 mM DTT, 0.5% Sodium deoxycholate, 10 mM Benzamidine, pH

8.0). The supernatant was collected from the homogenate, incubated in ice for 30 min and centrifuged at 13,000 g at 4°C for 15 min. Total protein was quantified by bicinchoninic acid kit (BCA Protein Assay Reagent Kit, Pierce).

Liposome-mediated extraction of PS from atherosclerotic plaques

Two liposome preparations,¹⁶ phosphatidylserine/phosphatidylcholine 80/20% (Lip_{80/20}) and 100/0% (Lip₁₀₀), or Hepes buffer (Hepes 20 mM, NaCl 150 mM, CaCl₂ 5 mM, pH 7.4) were used to draw PS from plaque samples, followed by Western blotting analysis conducted by 4-12% SDS-PAGE separation and polyclonal anti-PS antibody (Dako, Glostrup, Denmark), recognizing free and C4BP-bound PS.

Lipoproteins separation and PS content analysis

Apolipoprotein B100-bound VLDL and LDL (ApoB100_{VLDL/LDL}) and SAA2-containing HDL (SAA2_{HDL}) were obtained from fresh plasma samples of fasting TEA-P and HS on microplates by using goat polyclonal anti-ApoB100 (Sigma-Aldrich, Milan, Italy) and mouse polyclonal anti-SAA2 (Abnova, Taipei, Taiwan) antibodies, respectively. As a background study, plasma samples of TEA-P and HS were in parallel incubated on microplates with rabbit polyclonal anti-BSA antibody (Sigma-Aldrich, Milan, Italy). Microplate bound lipoproteins (SAA2_{HDL} and ApoB100_{VLDL/LDL}) were detached by LDS Sample Buffer (ThermoFisher, Milan, Italy) and analyzed for the presence of PS by Western blotting. Densitometric analysis of Western blots defined the amounts of PS bound to SAA2_{HDL} and to ApoB100_{VLDL/LDL} (PS-SAA2_{HDL} and PS-ApoB100_{VLDL/LDL}) after background subtraction and comparison with purified PS standard.

Statistics

Unpaired t test analysis (Welch's correction indicated by *) and Pearson's correlation were made by Graph Pad 8 (Prism) and SPSS statistics 25 (IBM). Data were analyzed before and after logarithmic transformations of values. A p value < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

We investigated the presence of PS in the carotid atherosclerotic plaque of patients that underwent endarterectomy (TEA-P), a surgical procedure to remove the atheromatous plaque material.

Plasma levels of PS and lipoproteins were compared between TEA-P and a group of healthy subjects (HS).

TEA-P did not differ from HS in PS plasma levels (TEA-P, 55.2±10.0 mg/L; HS, 52.5±14.9 mg/L) and HDL

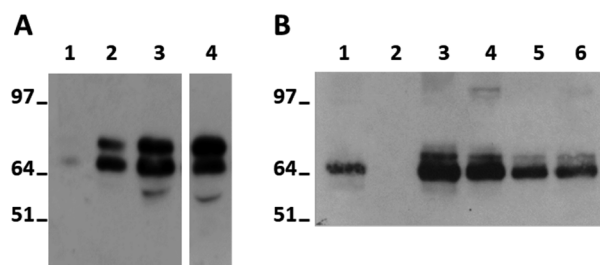
concentration (TEA-P, 57.2 ± 13.4 mg/dL; HS, 49.4 ± 14.5 mg/dL, $p=0.228$), and showed higher LDL concentration as a trend (TEA-P, 134.9 ± 90.5 mg/dL; HS, 81.0 ± 33.2 mg/dL, $p^*=0.104$). An inverse, although not significant, correlation between PS concentration and HDL levels was observed in HS ($r=-0.609$, $p=0.061$), but not in TEA-P ($r=0.021$, $p=0.955$).

The analysis of the lysed necrotic core of atherosclerotic plaque, tested via Western blotting, clearly showed the presence of increasing amounts of PS (Figure 1A) in direct relation to the increasing amounts of lysed tissue (lanes 1-3). Band size and pattern were comparable to that of PS in plasma (lane 4), albeit the larger and uncleaved form was more represented in plasma than in plaque.

Taking into account that the PS anticoagulant activities cannot be evaluated in lysed tissue, we investigated as surrogate the lipid binding properties of PS, experimentally released from the plaque after exposure of the necrotic core fraction to liposomes with different lipid composition and, as negative control, in the absence of liposomes (Figure 1B, lane 6). The amounts of PS recovered from the necrotic core in the presence of liposomes rich in phosphatidylserine (lane 4, Lip_{80/20}), for which PS affinity is well known, were much higher than those obtained with phosphatidylcholine liposomes (lane 5, Lip₁₀₀), which in turn were similar to those obtained with buffer (lane 6).

These experiments, which confirm the presence of measurable amounts of PS forms in atherosclerotic plaques, indicate that a portion of PS protein still maintains the ability to bind phosphatidylserine micelles.

Given the main role of plasma lipoproteins in lipid



Western blotting of PS in the necrotic core from carotid artery plaques were conducted by 4-12% SDS-PAGE separation and polyclonal anti-PS antibody. (A) Reducing conditions. Lanes 1, 2 and 3, increasing amounts (3, 10 and 30 μ g of total protein extract, respectively) of lysate necrotic core from plaque. Lane 4: human pooled normal plasma (PNP) diluted (1:5) to contain 100 ng of PS in 20 μ L. Migration is compatible with the entire (upper band) and cleaved (lower band) PS forms. (B) Non-reducing conditions. Equal portions (2 mg) of necrotic core were incubated overnight with liposomes (Lip_{80/20}, lane 4 and Lip₁₀₀, lane 5) or Hepes buffer (lane 6) at 4°C, and supernatants tested for the presence of PS. Lane 1, 30 ng of purified PS (HTI); lane 3, diluted (1:5) human PNP. Lane 2: 20 μ L of 10 mM Lip_{80/20} as a negative control.

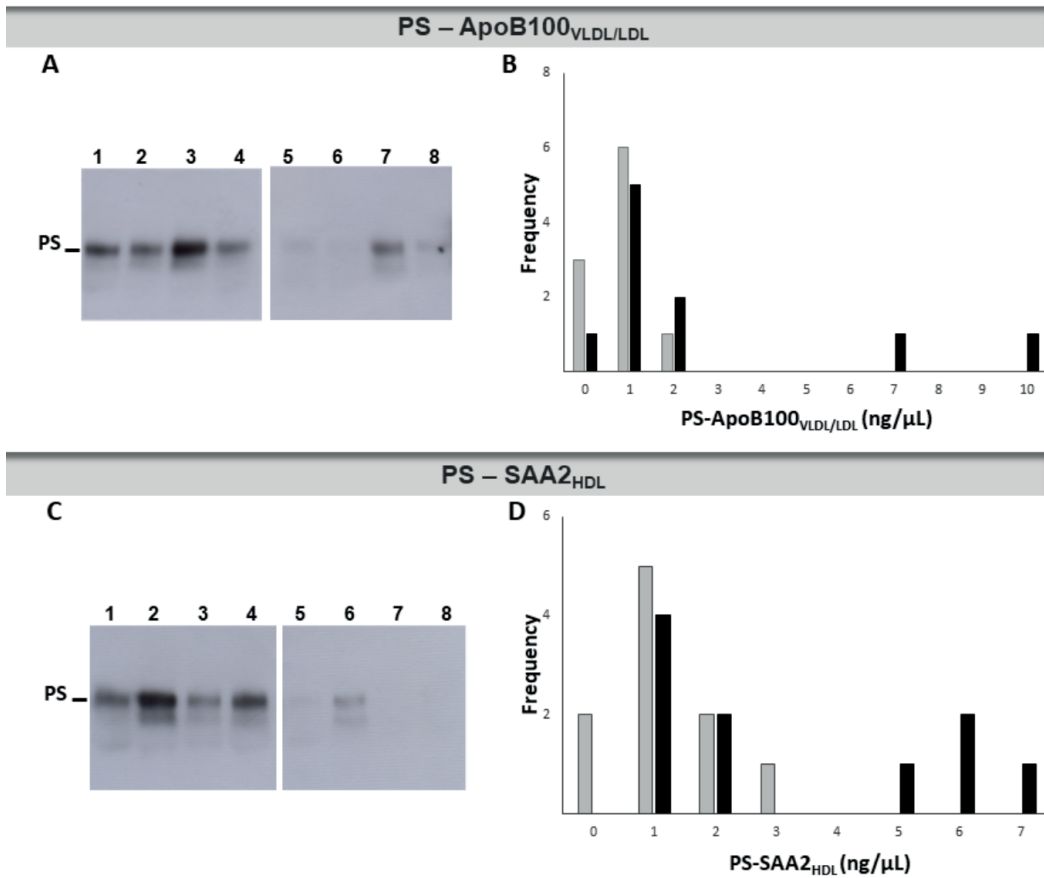
Figure 1. PS in atherosclerotic plaques by Western blotting analysis.

deposition into the plaque, the presence of PS on lipoproteins from fresh plasma was investigated. Lipoproteins were recovered by immunodepletion of plasma and subjected to Western blotting for PS (Figure 2A, lanes 1-4; Figure 2C, lanes 1-4). To support proper quantification of PS specifically bound to lipoproteins, we also measured the amount of PS not specifically bound to the microplate. This background, as reported in figures 2A (lanes 5-8) and 2C (lanes 5-8), was considered to quantify the amounts of bound PS. Comparison by densitometric analysis of samples from TEA-P and, as a reference, from HS is reported in figures 2B and 2D. Large variations in PS concentration were observed in VLDL/LDL captured by an anti-ApoB100 antibody in plasma from TEA-P (Figure 2B, 2.49 ± 3.19 ng/ μ L PS-ApoB100_{VLDL/LDL}, black bars) as compared with that from HS (0.87 ± 0.59 ng/ μ L, grey bars). The highest PS concentrations (>2 ng/ μ L) were detectable in VLDL/LDL from TEA-P. Noticeably, a positive correlation between PS-ApoB100_{VLDL/LDL} and LDL levels was detectable in TEA-P ($r=0.921$, $p<0.001$, Figure 3A, black dots), but not in HS (grey dots). The wide differences between TEA-P and HS in patterns of PS bound to lipoproteins are not based on different concentration and distribution of LDL, which were similar in the groups.

The analysis of PS was extended to HDL by the immunodepletion approach with an anti-SAA2 antibody, aimed at capturing the inflammatory HDL fraction. This approach could allow to evaluate the PS bound to these lipoproteins (Figure 2C and D). Densitometric analysis of Western blots showed higher amounts ($p^*=0.024$) of bound PS in TEA-P (3.19 ± 2.37 ng/ μ L) than in HS (1.13 ± 0.81 ng/ μ L), with the highest concentrations present in patients. PS bound to SAA2, and thus to inflammatory HDL, correlated as a trend with HDL concentration in plasma in TEA-P ($r=0.547$, $p=0.102$, Figure 3B), but not in HS ($r=-0.105$, $p=0.773$, Figure 2F). PS-SAA2_{HDL} in patients and HS did not correlate with PS plasma levels ($r=0.032$, $p=0.929$ and $r=0.016$, $p=0.965$, respectively).

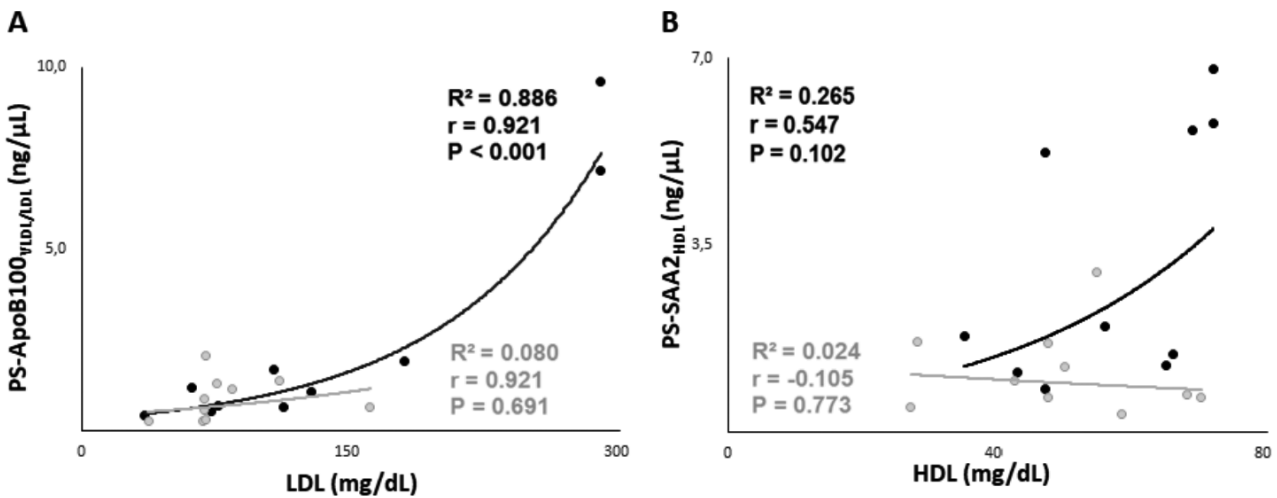
Interestingly, a positive correlation ($r=0.650$, $p=0.042$) was observed between levels of PS-SAA2_{HDL} and PS-ApoB100_{VLDL/LDL} in patients but not in HS ($r=-0.031$, $p=0.933$). Correlation between plasma PS concentration and PS-ApoB100_{VLDL/LDL} was not detectable in either TEA-P ($r=-0.241$, $p=0.502$) or HS ($r=0.479$, $p=0.161$).

The positive correlation between PS-bound and lipoprotein levels, detectable only in patients, suggested that specific features of lipoproteins in this group could be a key factor for the PS interaction. We are tempting to speculate that the significant correlation between PS-ApoB100_{VLDL/LDL} and LDL may reflect the specific lipid composition/oxidation of LDL in atherosclerotic patients. The undetectable correlation between amounts of PS bound to lipoproteins and plasmatic PS levels may support this hypothesis.



(A,C) Representative Western blots by polyclonal anti-PS on lipoproteins ApoB100_{VLDL/LDL} (A) or SAA2_{HDL} (C), captured in microplate from fresh plasma of fasting TEA-P (lanes 1-4, A and C). The non-specific binding of plasma PS to microplate (background, lanes 5-8, A and C), was subtracted in the densitometric analysis. (B, D) Densitometric analysis of PS-Western blots of ApoB100_{VLDL/LDL} (B) or SAA2_{HDL} (D) from plasma of HS (grey bars) or TEA-P (black bars), which showed large variation in PS concentration.

Figure 2. Western blotting analysis on lipoprotein-bound PS, in patients (TEA-P) and in healthy subjects (HS).



Correlation between lipoprotein-bound (A) PS-ApoB100_{VLDL/LDL} or (B) PS-SAA2_{HDL} with plasma levels of LDL or HDL, respectively, in TEA-P (black circles) and in HS (grey circles). Significant and positive correlation occurs only between PS-ApoB100_{VLDL/LDL} and plasmatic LDL levels in TEA-P (A). No correlation occurs between PS-SAA2_{HDL} and HDL levels, both in TEA-P and HS (B).

Figure 3. Correlation analysis between lipoprotein-bound PS and lipoprotein levels in plasma.

Overall data support the presence of PS protein in highly - one order of magnitude - variable amounts on the surface of lipoproteins, in particular on LDL from patients. The positive correlation with the plasma concentration of these micelles, and not with PS plasma concentration, suggests a role of the lipoprotein features for binding. On the other hand, it may be hypothesized that the PS-phospholipids binding may increase by oxidative processes.¹² The role of oxidized-LDL in atherosclerotic plaque formation and growth is well described,¹⁷ together with the differentiation into foam cells of macrophage and smooth muscle cells (SMCs) after LDL interactions.¹⁸ Furthermore SMCs, which are the major cell type in arterial wall with a pivotal role in plaque development, are able to synthesize and secrete PS with anticoagulant function *in vitro*.¹⁹ The results of the present study are also consistent with the concept of a deep, and still uncharted, link between plasma lipids/lipoproteins and the molecular players of coagulation pathway in patients with cardiovascular diseases.²⁰

CONCLUSIONS

Our study has some limitations. A small number of patients and healthy subjects, not matched for age, was compared, also related to the need of using fresh plasma and plaque samples for optimal lipoprotein isolation and PS extraction, respectively, in parallel assays. In addition, PS analysis was not performed in all plasma HDLs, but only on the SAA2-bound fraction, which anyway represents the major HDL subfraction during inflammation.² Finally, our data do not allow to establish whether a major part of the PS into atherosclerotic plaques derives from plasma or SMCs near to the necrotic core of the plaque, which deserves further investigation.

Despite these limitations, the delivery of the plasma PS into the necrotic core of the atherosclerotic plaques through lipoproteins, particularly the LDL, could be a physiopathological process with a plausible protective role during atherosclerosis progression and complications, like unstable plaque rupture, in which the anticoagulant properties of PS may potentially limit the superimposed thrombosis. However, the ability of PS to influence and limit plaque related thromboembolic events requires further investigation.

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