A novel prothrombotic role of proprotein convertase subtilisin kexin 9: the generation of procoagulant extracellular vesicles by human mononuclear cells

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Abstract
Background Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease synthesized mainly in the liver that binds the receptor of low-density lipoprotein and promotes its degradation in lysosomes. PCSK9 is considered a promising target for the development of new therapies for the treatment of hypercholesterolemia and related cardiovascular diseases. Extracellular vesicles represent a heterogeneous population of vesicles, ranging in size between 0.05 and 1 μm involved in numerous pathophysiological processes, including blood coagulation. We investigated whether PCSK9 stimulation induces the release of procoagulant extracellular vesicles from human mononuclear cells (PBMCs) and THP-1 cells.

Methods and results PBMCs and THP-1 cells were stimulated with PCSK9, the generation of EV was assessed by the prothrombinase assay and by cyttofluorimetric analysis. EV-associated tissue factor activity was assessed by a one-stage clotting assay. PCSK9 induced an increase in extracellular generation by PBMCs and THP-1 cells as well as an increase in extracellular vesicle-associated tissue factor. Pre-treatment with inhibitors of the toll like receptor, TLR4 (C34), and of NF-κB signaling (BAY 11-7082), downregulated PCSK9-induced extracellular vesicle generation and of extracellular-bound tissue factor. Similar effect was obtained by an anti-PCSK9 human-monoclonal antibody.

Conclusions PCSK9-mediated generation of procoagulant EV could contribute to increase the prothrombotic status in patients with cardiovascular diseases.

Keywords PCSK9 · Extracellular vesicles · Tissue factor · Procoagulant activity

Introduction
Proprotein convertase subtilisin kexin 9 (PCSK9) is a serine protease synthesized in the form of a solublezymogen mainly in the liver which, following an auto-catalytic process in the endoplasmic reticulum, is transformed into proprotein convertase. PCSK9 is secreted into the bloodstream, where it binds the low-density lipoprotein (LDL) receptor in its extra-cellular portion and its degradation products in lysosomes, preventing its recycling on the hepatocyte membrane [1]. PCSK9 gain-of-function mutations are associated with autosomal dominant hypercholesterolemia and premature atherosclerosis [2]. PCSK9 loss-of-function mutations conversely lead to low levels of LDL-cholesterol and cardiovascular protection [3]. Pharmacological inhibition of PCSK9 with monoclonal antibodies leads to a 60% LDL-cholesterol reduction in patients already treated with maximum tolerated statin therapy [4]. High circulating levels of PCSK9 predict cardiovascular events in patients with atrial fibrillation and in those with stable coronary artery disease (CAD), including patients with well-controlled LDL-cholesterol levels [5].

Tissue factor (TF) is an integral membrane protein constitutively expressed in subendothelial tissues and exposed to the blood following injury. TF is a high affinity receptor and essential cofactor for coagulation factor (F) VII(a); upon binding to TF, FVII(a) activates both FX and FIX thus...
initiating a series of reactions that eventually lead to fibrin generation [6]. An inducible form of TF is also synthesized by blood and vascular cells upon stimulation by different agonists. Circulating monocytes are endowed with a potential for synthesizing and expressing substantial amounts of TF [6]. Quite notably, monocytes represent the effector arm of the innate immune system deeply involved in endogenous inflammatory processes [7] and an extensive crosstalk links these two systems, whereby inflammation activates coagulation and coagulation also considerably affects inflammatory activity [8]. Lipopolysaccharide (LPS, endotoxin) is one of the best characterized agonists capable of stimulating TF synthesis by monocytes. LPS is the principal glycolipid component of the outer membrane of Gram-negative bacteria and a well-characterized inflammatory stimulus, signaling through the Toll-like (TL) receptor pathway [9].

Extracellular vesicles (EV) are cell membrane-derived vesicles ranging between 0.1 and 1 µm in diameter shed upon activation and/or during apoptosis by virtually all cells including monocytes [10]. The discovery that EV could harbor functional TF [11] has contributed to modify the paradigm of circulating monocytes and endothelial cells as the only sources of intravascular procoagulant material released in response to inflammatory stimuli [12]. Exposure to LPS [13] as well as other agonists, including angiotensin II [14], rosiglitazone [15], environmental particulate matter [16] and leptin [17] causes an increase in procoagulant, monocyte-derived TF-bearing EV.

We recently provided evidence that PCSK9 stimulation elicits TF expression in cells of monocytic lineage through the activation of the TLR4/NF-κB signaling pathway [18]. Based on the above data and on the observation that both PCSK9 and EV are increased in thrombotic diseases [1, 19] we hypothesized that PCSK9 induces prothrombotic, TF-bearing EV generation by monocytes, possibly via TLR4/NF-κB activation.

### Materials and methods

#### Reagent and materials

RPMI-1640, penicillin, streptomycin, trypsin blue, Histopaque®-1077, sodium citrate, LPS from *Escherichia coli* O55:B5, hrPCSK9, BAY 11–7082 (BAY), β-mercaptoethanol and Fetal bovine serum (FBS) were obtained from Sigma-Aldrich (Milan, Italy). Human relipidated full length recombinant human TF was from BioMedica Diagnostics (Windsor, Canada). Ultrapure LPS-RS, CLI-095 (CLI) were purchased from InvivoGen (San Diego, California). The human monoclonal antibody anti-PCSK9 was a generous gift from Amgen Inc. C34 was purchased from Tocris Bioscience (Bristol, UK). LAL chromogenic endpoint assay was obtained from Hycult Biotech (Uden, The Netherlands). Annexin V was purchased by Biosciences (Dublin, Ireland). Carboxyfluorescein succinimidyl ester (CFSE) was purchased by Abcam (Cambridge, UK). THP-1 cells were from the European Collection of Authenticated Cell Cultures (ECACC).

#### Cell cultures

Human peripheral blood mononuclear cells (PBMCs) were obtained as described [18]. Briefly, suspensions derived from single-donoruffy coats were obtained from the local blood bank and represented leftovers otherwise destined to disposal. The procedure was approved by the local ethics committee. Theuffy coats were kept at room temperature and utilized within 4 h from withdrawal. PBMCs were isolated by centrifugation of fresh buffy coats on Histopaque®-1077 at 400×g for 30 min at 20 °C. Cells collected at the interphase were washed twice in sodium citrate 0.38% and resuspended in RPMI-1640 supplemented with 1% penicillin–streptomycin. The final PBMCs preparations typically contain 25–35% monocytes, negligible proportions of neutrophils (<5%), 65–75% lymphocytes and some contaminating platelets. Cell viability was assessed by dimethyl thiazolyl diphenyl tetrazolium (MTT) (Sigma-Aldrich, Milan, Italy): high viability (>85%) was confirmed for all preparation. To account for interindividual variability among donors as well as interferences from copurified lymphocyte- and contaminant platelet-derived TF, cells of the monocytic lineage, THP-1, were also used in selected experiments. Cells were cultured in RPMI 1640 medium supplemented with 10% heat-inactivated FBS, 5% β-mercaptoethanol, 2 mM l-glutamine, 50 U/mL penicillin, and 50 g/mL streptomycin and incubated in a 5% CO2 humidified atmosphere at 37 °C. Throughout the study procedures, THP-1 cells were maintained in a logarithmic growth phase at a concentration between 3 and 5 × 10⁵ cells/mL.

Cell cultures suspended in polypropylene tubes, PBMCs (3 × 10⁶ cells/mL) and THP-1 (1 × 10⁶ cells/mL), were incubated with PCSK9 (1 and 5 µg/mL; 18 and 4 h for the two cell types, respectively) prior to EV-associated TF-PCA and total EV analysis. LPS at concentrations of 0.1 and 10 µg/mL for PBMCs and THP-1 respectively was used as a positive control in all set of experiments. Antagonists were added to cell culture 30 min prior to stimulation.

To prevent LPS contamination, glassware was exposed to high temperature (200 °C for 4 h). In addition, plasticware, reagents and solutions used for in vitro cell cultures were preliminarily tested on a routine basis with a
sensitive chromogenic LAL assay. Reagents with endotoxin concentrations > 60 pg/mL were discarded.

**Assessment of EV generation**

EV generation was investigated by two independent methods. The Zymuphen MP-activity kit (Hyphen BioMed, Neuville-sur-Oise, France) measures the concentration of phosphatidylserine (PS) based on the rate of prothrombin conversion to thrombin in a solid-phase chromogenic assay in which the availability of PS is the rate limiting step [20]. Thrombin generation is then converted to PS concentration by mean of a standard curve generated with known concentrations of PS, according to the manufacturer’s instructions. PS concentration was assessed in the cell-free conditioned medium of PBMC and THP1 cells, after culture in different conditions. Under these experimental conditions, EV are considered the only source of PS. A cyttofluorimetric analysis was conducted using a FACSscan™II flow cytometer (BD Biosciences, San Jose, CA, USA). EV were first discriminated by size, using commercially available calibration beads (Megamix Plus—SSC; Stago, Milan, Italy), as events conforming to a light scatter distribution within the 0.16−0.5 μm range in a SSC vs. FSc window and further identified as annexin V⁺, to identify medium-large EV expressing phosphatidylserine [10], and CFSE⁺ events after incubation with allophycocyanin (APC)-annexin V and CFSE, in a APC vs. FITC window. Annexin-V binds to PS and is therefore used as a marker for PS-exposing EV. CFSE was used as a marker of EV integrity [21]. Events acquisition was obtained at low flow rate and stopped after 180 s.

**Assessment of EV-associated TF activity**

TF activity was measured in EV derived from PBMCs (3 × 10⁶ cells/mL) and THP-1 cells (1×10⁶ cells/mL) by a one-stage clotting time test using a STart Max semi-automated Coagulation analyzer (Diagnostica Stago S.A.S., Milan, Italy). The conditioned media were cleared by centrifugation at 16,000xg for 5 min at 4° to remove dead cells and big cell fragments that might have detached, and then submitted to further centrifugation at 16,000xg for 45 min at 4 °C. The pellets were resuspended in 125 μL normal saline, lysed by three freeze–thaw cycles and added to an equal volume of pooled, EV-free normal human plasma. Time to clot formation upon recalcification with 25 mM CaCl₂ was recorded. All experiments were carried out at 37 °C. For each experimental session, calibration curves were generated using recombinant human relipidated TF (pg/mL) (BioMedica Diagnostics-Windsor-NS Canada). In our experimental conditions, clotting times of 627 ± 84 s and 19 ± 2 s (mean ± SD) were obtained with 0.001 pg/mL and 100 pg/mL TF, respectively. Experiments were run in triplicate and averaged.

**Statistical analysis**

Data are shown as mean ± SEM. Comparisons between two groups were performed using unpaired t-test; comparisons among more than two groups were performed using ANOVA for independent measures followed by Sidak’s post-hoc analysis. All tests are two-tailed. Prism software (Graph-Pad, San Diego, CA, USA) was used for analysis and graph generation.

**Results and discussion**

First, we investigated whether PCSK9 stimulation induces EV generation by monocytic cells. As shown in Fig. 1A, incubation of PBMCs with PCSK9 (1 μg/mL) causes a significant increase in procoagulant EV generation, as assessed by an assay that measures phosphatidylserine (PS) concentration. Similar results were obtained with cells of the monocytic line, THP-1 (Fig. 1B). To confirm the specificity of the phenomenon, we used evolocumab, an inhibitory human
monoclonal antibody directed against PCSK9. The antibody abolished the upregulation of EV generation by PBMC after incubation with PCSK9 (Fig. 1C).

We next investigated some of the mechanisms of PCSK9 induced upregulation of EV generation. BAY-117082 (BAY), a compound which inhibits NF-κB by preventing the phosphorylation and subsequent degradation of IκB abolished EV generation upon PCSK9 stimulation both in PBMCs and THP-1 (Fig. 2A, B, respectively). A similar inhibitory effect was induced by the TLR4 inhibitor, C34 (Fig. 2C).

The results described above were obtained analyzing the concentration of PS in the conditioned medium. As this medium is cell-free, EV are likely the only source of PS. However, to confirm the results we also analyzed EV by flow cytometry. EV were defined as events conforming to light scatter distribution within the 0.16–0.5 μm bead range in a SSc vs FSc window and further identified as annexin V positive events, and therefore expressing PS, and carboxyfluorescein diacetate succinimidyl ester (CFSE) positive events. CFSE is a membrane permeant molecule that, upon cleavage by intracellular esterases, produces a fluorescent dye. Accordingly, CFSE positivity confirms that the events detected are closed vesicles with an intact membrane, enclosing esterases rather than cell debris. With this approach, we also confirmed that PCSK9 induces the release of EV by THP-1 (235 ± 18 events SSC+/annexinV+/CFSE+ in basal condition vs 565 ± 122 events SSC+/annexinV+/CFSE+ after stimulation with PCSK9; mean ± SD, p < 0.05).

The EV generated upon PCSK9 stimulation are procoagulant, due to the exposure of PS, a negatively charged phospholipid required for the assembly of the multimolecular complexes that participate in the coagulation cascade. However, EV also might contribute to the coagulation processes through the exposure of TF [12]. Indeed, EV generated by both PBMC and THP1 cells upon stimulation with PCSK9 showed a TF-dependent procoagulant activity as assessed by a one-stage clotting assay (Fig. 3A, B, respectively). Again, direct PCSK9 inhibition with evolocumab and blocking of the of NF-κB-dependent signaling pathway by BAY abrogated PCSK9-induced generation of EV-bound TF-dependent procoagulant activity both in PBMCs (Fig. 3C) and THP-1 cells (not shown).

![Fig. 2](image-url)  
**Fig. 2** Inhibition of EV generation, expressed as PS concentration, by PBMCs (A) and THP-1 (B, C) by BAY-117082 (A, B) and C34 (C). * and ***p < 0.05 and 0.001, respectively, for inhibited versus uninhibited; n = 5 (A, B) and 4 (D). ANOVA with Sidak’s post-hoc analysis

![Fig. 3](image-url)  
**Fig. 3** Effect of PCSK9 on the induction of TF-dependent procoagulant activity by PBMC (A) and THP-1 (B). ** and ***p < 0.01 and 0.001, respectively, n = ; Student’s t-test. C Inhibition of PCSK9 induced TF-dependent procoagulant activity by evolocumab and BAY117082. ***p < 0.001; n = 5 (A, C), and 8 (B); ANOVA followed by Sidak’s post-hoc analysis
Our data indicate that PCSK9 induces the generation of prothrombotic, TF-bearing EV by cells of monocytic lineage. A specific monoclonal antibody to PCSK9, evolocumab, blocked the phenomenon, confirming its specificity and effectively ruling out, for example, a potential role of LPS contamination of the reagents. Inhibition with BAY-117982 and C34 suggest that PCSK9-induced stimulation of EV shedding is mediated through NF-κB and TLR4.

The role of EV in numerous pathophysiologic phenomena has gained much attention over the last several years. Specifically in the field of coronary heart diseases, increased numbers of EV have been demonstrated in the peripheral blood of patients with acute coronary syndromes; this observation has been linked, at least in part, to the procoagulant role of PS and TF on their surface [12].

PCSK9 has been extensively studied for its role as a key regulator of cholesterol metabolism [1]. However, it has become clear over the years that the effects of this molecule in the pathogenesis of atherothrombosis are more complex. Indeed, PCSK9 promotes vascular inflammation through TLR4/NF-κB-mediated modulation of proinflammatory cytokine release [22], an activity that this molecule shares with LPS [23].

We have recently shown that PCSK9 also induces TF expression by monocytes, again via a TLR4/NF-κB pathway [18]; this observation adds to the general understanding of PCSK9 as an agonist with pleiotropic proinflammatory and prothrombotic effects. Our current data further expand on the same line.

The analysis of EV is notoriously complex and none of the available methods (that include, among others, flow cytometry, nanotrack analysis, electron microscopy, PS analysis) is considered sufficient to clearly identify and enumerate specific vesicles [10]. In the current work we have used two independent approaches. One approach measures PS concentration based on the kinetics of thrombin generation in a chromogenic assay in which PS is the rate limiting component. The approach has the advantage of being insensitive to the dimensions of the EV but does not distinguish between EV and cell fragments. In contrast, flow cytometry does recognize closed vesicles enclosing intact cytoplasm via the use of CFSE, a molecule that fluoresces only upon cleavage by intracellular esterases; flow cytometry, however, does not recognize events smaller than approximately 200 nm in size [24, 25]. The use of both approaches increases the reliability of the results.

We had originally planned to perform most experiments with PBMC in parallel with those with THP-1; however, the SARS-CoV2 pandemic has prevented us from accessing blood-derived products for research purposes.

In conclusion, we demonstrate that PCSK9 might contribute to the atherothrombotic process through the induction of EV expressing both PS and TF and therefore capable of activating the coagulation cascade. Further studies will investigate more rigorously the mechanisms of PCSK9-induced EV generation.

Author contributions The study conception was performed by AC, CS, DN, VS and TN. Material preparation, data collection and analysis were performed by SL, VS and TN; The first draft of the manuscript was written by AC, RP, VS and TN and. All authors read and approved the final manuscript.

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Declarations

Conflict of interest The authors have no relevant financial or non-financial interests to disclose.

Ethical approval The study was conducted according to the guidelines of the Declaration of Helsinki and approved by the Ethics Committee of the Pisa University Hospital (Protocol code 558).

Consent to participate Buffy coats obtained from healthy donors used for this study were, “waste of the sample examined” not used for the dosage of biochemical analytes. Data security was maintained by the impossibility of tracing the patient’s identity and, therefore, any further information.

References

1. Barale C, Melchionda E, Morotti A, Russo I (2021) PCSK9 biology and its role in atherothrombosis. Int J Mol Sci 22:5880
2. Abifadel M et al (2003) Mutations in PCSK9 cause autosomal dominant hypercholesterolemia. Nat Genet 34:154–156
3. Cohen JC, Boerwinkle E, Mosley TH, Hobbs HH (2006) Sequence variations in PCSK9, low LDL, and protection against coronary heart disease. N Engl J Med 354:1264–1272
4. Navarese EP, Kołodziejczak M, Dimitroulis D, Wolff G, Busch HL, Devito F, Sionis A, Ciccone MM (2016) From proprotein convertase subtilisin/kexin type 9 to its inhibition: state-of-the-art and clinical implications. Eur Heart J Cardiovasc Pharmacother 2:44–53
5. Cao YX, Jin JL, Sun D, Liu HH, Guo YL, Wu NQ, Xu RX, Zhu CG, Dong Q, Sun J, Li JJ (2019) Circulating PCSK9 and cardiovascular events in FH patients with standard lipid-lowering therapy. J Transl Med 17:367
6. Camerer E, Kolsto AB, Pryds H (1996) Cell biology of tissue factor, the principal initiator of blood coagulation. Thromb Res 81:1–41
7. Ghantas A, Griffiths HR, Devitt A, Lip GYH, Shantsila E (2013) Monocytes in coronary artery disease and atherosclerosis. J Am Coll Cardiol 62:1541–1551
8. Levi M, van der Poll T, Bülter HR (2004) Bidirectional relation between inflammation and coagulation. Circulation 109:2698–2704
9. Beutler B, Hoebe K, Du X, Ulevitch RJ (2003) How we detect microbes and respond to them: the toll-like receptors and their transducers. J Leukoc Biol 74:479–485
10. Théry C et al (2018) Minimal information for studies of extracellular vesicles 2018 (MISEV2018): a position statement of the international society for extracellular vesicles and update of the MISEV2014 guidelines. J Extra Vesicles 7:1461450
11. Falati S, Liu Q, Gross P, Merrill-Skoloff G, Chou J, Vandendries E, Celi A, Croce K, Furie BC, Furie B (2003) Accumulation of tissue factor into developing thrombi in vivo is dependent upon microparticle P-selectin glycoprotein ligand 1 and platelet P-selectin. J Exp Med 197:1585–1598
12. Owens AP, Mackman N (2011) Microparticles in hemostasis and thrombosis. Circ Res 108:1284–1297
13. Bernimoulin M, Waters EK, Foy M, Steele BM, Sullivan M, Falet H, Walsh MT, Barteneva N, Geng JG, Hartwig JH, Maguire PB, Wagner DD (2009) Differential stimulation of monocytic cells results in distinct populations of microparticles. J Thromb Haemost 7:1019–1028
14. Cordazzo C, Neri T, Petrini S, Lombardi S, Balia C, Cianchetti S, Carmazzi Y, Paggiaro P, Pedrinelli R, Celi A (2012) Angiotensin II induces the generation of procoagulant microparticles by human mononuclear cells via an angiotensin type 2 receptor-mediated pathway. Thromb Res 131:e168–e174
15. Neri T, Cordazzo C, Carmazzi Y, Petrini S, Balia C, Stefanelli F, Amoroso A, Brunelleschi S, Breschi MC, Pedrinelli R, Paggiaro P, Celi A (2012) Effects of peroxisome proliferator-activated receptor-gamma agonists on the generation of microparticles by monocytes/macrophages. Cardiovasc Res 94:537–544
16. Neri T, Pergoli L, Petrini S, Gravendonk L, Balia C, Scalise V, Amoroso A, Pedrinelli R, Paggiaro P, Bollati V, Celi A (2016) Particulate matter induces prothrombotic microparticle shedding by human mononuclear and endothelial cells. Toxicol In Vitro 32:333–338
17. Petrini S, Neri T, Lombardi S, Cordazzo C, Balia C, Scalise V, Paggiaro P, Pedrinelli R (1860) Celi A (2016) Leptin induces the generation of procoagulant, tissue factor-bearing microparticles by human peripheral blood mononuclear cells. Biochim Biophys Acta 6:1354–1361
18. Scalise V, Sanguinetti C, Neri T, Cianchetti S, Lai M, Carnicelli V, Celi A, Pedrinelli R (2021) PCSK9 induces tissue factor expression by activation of TLR4/NFκB signaling. JMS 22:12640
19. Giró O, Jiménez A, Pané A, Badimon L, Ortega E, Chiva-Blanch G (2021) Extracellular vesicles in atherothrombosis and cardiovascular disease: friends and foes. Atherosclerosis 330:61–75
20. Neri T, Lombardi S, Faita F, Petrini S, Balia C, Scalise V, Paggiaro P, Celi A (2016) Pirfenidone inhibits p38 mediated generation of procoagulant microparticles by human alveolar epithelial cells. Pulm Pharmacol Ther 39:1–6
21. Neri T, Scalise V, Passalacqua I, Giusti I, Lombardi S, Balia C, D’Alessandro D, Berrettini S, Pedrinelli R, Paggiaro P, Dolo V, Celi A (2018) CD18-mediated adhesion is required for the induction of a proinflammatory phenotype in lung epithelial cells by mononuclear cell-derived extracellular vesicles. Exp Cell Res. https://doi.org/10.1016/j.yexcr.2018.02.023
22. Guijarro-Muñoz I, Compte M, Álvarez-Cienfuegos A, Álvarez-Vallina L, Sanz L (2014) Lipopolysaccharide activates toll-like receptor 4 (TLR4)-mediated NF-xB signaling pathway and proinflammatory response in human pericytes. J Biol Chem 289:2457–2468
23. Luquero A, Badimon L, Borrell-Pages M (2021) PCSK9 functions in atherosclerosis are not limited to plasmatic LDL-cholesterol regulation. Front Cardiovasc Med 8:639727
24. de Rond L, van der Pol E, Bloemen PR, Van Den Broeck T, Monheim L, Nieuwland R, van Leeuwen TG, Coumans FAW (2020) A systematic approach to improve scatter sensitivity of a flow cytometer for detection of extracellular vesicles. Cytometry A 97:582–591
25. Gomes J et al (2018) Analytical considerations in nanoscale flow cytometry of extracellular vesicles to achieve data linearity. Thromb Haemost 118:1612–1624

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