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ORIGINAL ARTICLE

Rosuvastatin treatment decreases plasma procoagulant phospholipid activity after a VTE: A randomized controlled trial

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Abstract

Background: Venous thromboembolism (VTE) is a frequent cardiovascular disease with severe complications, including recurrence and death. There is a great need for alternative prophylactic treatment options as anticoagulation is accompanied by increased bleeding risk. Statins are reported to reduce the risk of incident and recurrent VTE, but the mechanisms are elusive. Procoagulant phospholipids (PPL), and phosphatidylserine in particular, are crucial for efficient coagulation activation, but no studies have investigated the effect of statin treatment on plasma PPL activity.

Objectives: To investigate the impact of rosuvastatin treatment on plasma PPL activity and levels of extracellular vesicles (EVs).

Patients/Methods: Patients with a history of VTE (≥18 years) allowed to stop anticoagulant treatment were randomized to either 20 mg/day of rosuvastatin treatment or no treatment for 28 days in the Statins Reduce Thrombophilia (NCT01613794) trial. Plasma samples were collected at baseline and study end. PPL activity was measured in samples from 245 participants using a factor Xa-dependent clotting assay and EV levels by flow cytometry.

Results: Rosuvastatin treatment yielded an overall 22% (95% confidence interval [CI] −38.2 to −5.8) reduction in PPL activity, and 37% (95% CI −62.9 to −11.2) reduction in PPL activity in participants with a history of pulmonary embolism. The effect of rosuvastatin on plasma PPL activity was not explained by changes in total cholesterol nor change in levels of total- or platelet-derived EVs.

Conclusions: Rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that a PPL-dependent attenuation of coagulation activation may contribute to a reduced VTE risk following statin treatment.

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KEYWORDS
blood clotting, extracellular vesicles, phosphatidylserines, rosuvastatin, venous thromboembolism

1 | INTRODUCTION

Venous thromboembolism (VTE), comprising deep vein thrombosis (DVT) and pulmonary embolism (PE), is a frequent cardiovascular disease with severe short- and long-term complications, including recurrence and death.[1-3] At present, anticoagulation is the treatment of choice for primary and secondary prophylaxis of VTE.[4] Although highly efficient, anticoagulation is accompanied by increased bleeding risk, where 1–4% annually experience major bleeding events, depending on type of anticoagulant, dose, and duration of treatment.[5-7] As VTE recurs in up to 30–40% of patients within 10 years of the initial event,[8-11] there is a need for alternative prophylactic treatment options in patients with high bleeding risk.

HMG-CoA (3-hydroxy-3-methyl-glutaryl-CoA) reductase inhibitors, known as statins, are a class of cholesterol-lowering drugs with antithrombotic properties and preventive effect on VTE. Observational and randomized studies have reported a 14–54% reduction in the risk of a first VTE[12-16] and a 27–50% reduction in recurrent events[17-20] following statin treatment. However, there is only limited knowledge on the pleiotropic effects of statins that may explain their beneficial effects on the risk of VTE. The Statins Reduce Thrombophilia (START) trial was established to address this knowledge gap. Previous results from the START trial have shown that rosuvastatin treatment modestly reduced (3–6%) the plasma level of several coagulation factors,[21] in particular factor VIII, lowered the tissue factor (TF)-induced potential for thrombin generation in plasma by 10%,[22] and increased the fibrinolytic potential in plasma.[23] However, the beneficial effect of statins on VTE could not be explained by reduced platelet reactivity, measured by thromboxane-A2–mediated platelet aggregation.[24]

Negatively charged phospholipids, and phosphatidylserine (PS) in particular, are vital to coagulation. PS is located on the surface of activated platelets as well as on extracellular vesicles (EVs), and they facilitate the assembly of coagulation factors VII (FVII), FIX, FX, and prothrombin (FII)[25] in blood. The presence of negatively charged phospholipids augments the activity of the extrinsic tenase complex, TF-FVIIa, by several orders of magnitude.[26] The procoagulant phospholipid (PPL) activity of plasma samples can be measured using a factor Xa (FXa)-dependent clotting assay. Previously, an inverse correlation has been established between PPL, measured by FXa-dependant clotting assays and plasma levels of PS-positive (PS^+) EVs.[27,28] However, the effect of statin treatment on plasma PPL activity has previously not been investigated. In the present study, we aimed to (1) investigate the impact of rosuvastatin treatment on plasma PPL activity in individuals with a history of VTE in a randomized controlled trial, and (2) explore the effect of statin treatment on total- and platelet-derived EV counts using a sensitive flow cytometer.

ESSENTIALS

- The effect of statin treatment on plasma procoagulant phospholipid (PPL) activity is not known.
- We measured the PPL activity in plasma samples from the Statins Reduce Thrombophilia trial.
- Rosuvastatin treatment caused a 22% decrease in plasma PPL activity.
- The effect was not explained by changes in plasma levels of extracellular vesicles measured by flow cytometry.

2 | MATERIALS AND METHODS

2.1 | Trial design

The START trial (NCT01613794) is a multicenter, randomized, controlled, open label clinical trial aimed to investigate the impact of rosuvastatin treatment on the coagulation profile of individuals with a previous history of VTE. The study has been described in detail elsewhere.[21] In brief, participants were recruited from three Dutch anticoagulation clinics (Leiden, Hoofddorp, and Rotterdam) that monitor anticoagulant treatment of VTE patients within a geographical area. Subjects with confirmed initial or recurrent symptomatic proximal DVT or PE allowed to stop oral anticoagulation treatment by their treating physician and aged 18 years or older were invited to participate. Exclusion criteria were the following: individuals already using statins or other lipid lowering drugs, or if contraindications for 20 mg/day rosuvastatin use were present, based on information provided by the instruction leaflet of the drug manufacturer. Participants were randomly assigned to either 20 mg/day of rosuvastatin or no study medication for the 28-day study duration. Compliance to treatment was assessed by measurements of total cholesterol levels at baseline and at study end in all participants. The START trial was approved by the Medical Ethics Committee of the Leiden University Medical Center, Leiden, the Netherlands, and all study participants gave written informed consent prior to participation.

2.2 | Baseline measurements

The study baseline was set as the last regular visit of the participant to the anticoagulation clinic. All participants were screened on acquired risk factors for VTE through a questionnaire, in addition to being tested on kidney and liver function. Participants stopped using their vitamin K antagonist 1 month prior to study inclusion and

baseline blood draw to allow for a leaching period for the anticoagulant drugs. Non-fasting blood samples were collected in Vacutainer tubes containing 3.2% sodium citrate (Becton Dickinson) at baseline and at study end (i.e., 28 days later). Samples were centrifuged at 2500 *g* for 15 min at 18°C and platelet-poor plasma (PPP) was stored at −80°C until analysis.

2.3 | Measurement of PPL clotting activity in plasma

A modified FXa-dependent PPL clotting assay (in house), previously described in detail,[29] was used to measure plasma levels of PPL in citrated platelet-free plasma (PFP; $n = 245$). Briefly, PPP samples were thawed and centrifuged 13,500 *g* for 2 min to generate PFP. PPL-depleted plasma (PPLDP) was prepared from pooled citrated PFP (n = 18) centrifuged at 100,000 *g* for 60 min at 16°C (Beckman Optima LE-80K Ultracentrifuge, rotor SW40TI, Beckman Coulter). PPLDP was aliquoted and stored at −80°C until further use. Twenty-five µl of test plasma was mixed with 25 µl PPLDP, incubated for 2 min at 37°C, before the reaction was initiated by the addition of 100 µl of pre-warmed FXa reagent (0.1 U/ml bovine FXa in 15 mM calcium chloride, 100 mM sodium chloride, and 20 mM HEPES buffer, pH 7.0). A commercially available standardized reagent containing 0.1% of rabbit brain cephalin in a buffered solution was used as calibrator (UPTT from Bio/Data Corporation). Clotting tests were carried out in duplicate on a StarT4 instrument from Diagnostica Stago. PPL levels were measured in seconds of clotting time, and converted to mU/ml, using the UPTT calibrator. The PPL assay displayed low inter and intra coefficients of variability (CVs) of ≤4% and variation between runs was adjusted for by an internal standard.

2.4 | Analysis of total- and platelet-derived microvesicles in plasma by flow cytometry

Plasma samples were selected from 40 participants from the rosuvastatin treatment group and 20 participants from the non-statin group. Participants with the largest decrease in plasma PPL activity were selected from the rosuvastatin treatment group as measurement of microvesicles by flow cytometry was expected to be less sensitive for changes than the plasma PPL activity. One sample from the no treatment group was later excluded due to technical failure. Plasma samples were thawed and centrifuged a second time for 2500 *g* for 15 min. Two hundred microliter of PFP was diluted 10x in pre-filtered (Amicon Ultra-15 filters, 10 kDa cutoff) Dulbecco's phosphate-buffered saline (DPBS) that is free of Ca^{2+}/Mg^{2+} (Thermo Fisher Scientific). Samples were centrifuged at 20,000 *g* for 30 min at 4°C to pellet EVs. Supernatants were carefully aspirated and the EV pellets were divided and stained for PS using fluorescein isothiocyanate (FITC)-labeled bovine lactadherin (Haematologic Technologies) and CD41 APC-H7 clone HIP8 (BioLegend), or with FITC-labeled bovine lactadherin and matched isotype controls. All antibodies and isotype controls were filtered using 0.22 µm ultrafree-MC centrifugal filter (Merck, Millipore) before use. EV pellets were incubated with antibody or isotype control mixture for 20 min at 4°C in the dark. Samples were washed with 1 ml pre-filtered DPBS and centrifuged at 20,000 *g* for 30 min at 4°C. Pellets were resuspended in 200 µl pre-filtered DPBS and samples were analyzed using CytoFLEX (Beckman Coulter) at the rate of 10 µl/min. Data analysis was performed using CytExpert 2.0 (Beckman Coulter). The EV gate was set using Rosetta calibration beads (Exometry). EVs were defined according to size and lactadherin-positive staining. The total number of EVs was calculated from the number of detected lactadherin-positive events in every sample, and further converted to EV number per microliter plasma (EV/µl) using the original volume of analyzed plasma (150 µl).

2.5 | Statistical analysis

Statistical analyses were performed using R (version 4.0.3 for Windows; R Foundation). Descriptive statistics were used to describe the baseline difference between the intervention and the control group. For the results tables, the treatment and no treatment group, as well as subgroups, were compared using two-sample *t*-tests with equal variance assumed and standard multivariate linear regression models adjusting for age and sex. To minimize the effect of intra- and inter-individual variability of flow cytometry data on plasma EVs, these data were displayed as percent change from baseline before comparison between groups. Pearson's correlation coefficient was used to estimate correlation.

3 | RESULTS

3.1 | Study population

Between December 2012 and December 2016, 255 participants were randomized to either the rosuvastatin treatment group ($n = 131$) or the no treatment group ($n = 124$). A study flowchart is shown in Figure 1 with reasons for exclusion. Two participants did not start rosuvastatin treatment, and another six randomized participants did not complete the study, three in each study arm due to various reasons. The PPL assay measurements could not be performed in two participants due to technical failure, one in each study arm. Hence, our study population consisted of 125 participants in the treatment group and 120 in the no treatment group. The time between the acute VTE and study randomization varied between 4 to 14 months with a median of 7 months. The baseline characteristics of the study population are shown in Table 1. Participants allocated to no treatment were slightly older (mean age 59 years) compared to the statin users (mean age 57 years), and were more

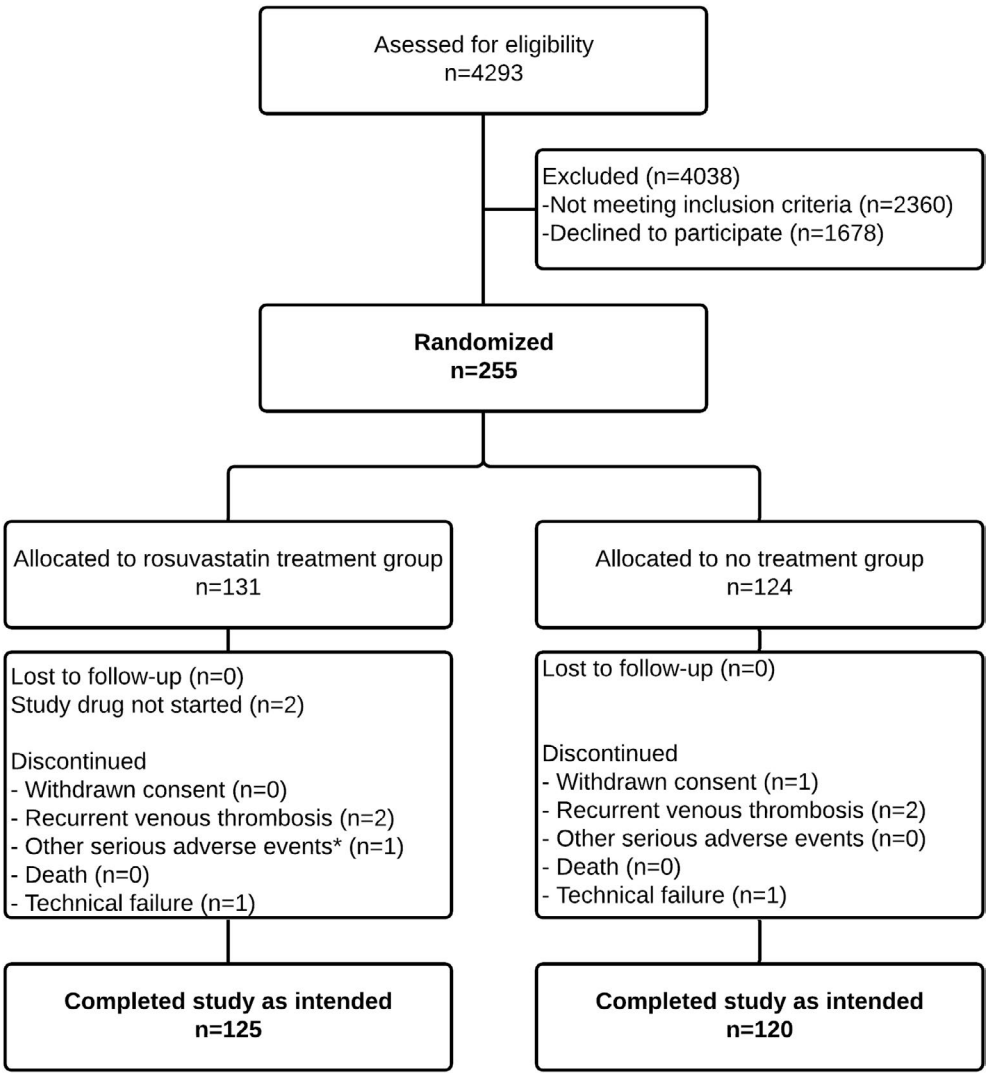

FIGURE 1 Flowchart of the study participants with numbers at enrolment, randomization, and follow-up, and reasons for withdrawal. *Hospitalization with acute asthma exacerbation

often male (69% vs. 54%). Other characteristics of the participants associated with VTE risk were equally distributed among the groups.

3.2 | Outcomes

Table 2 and Figure 2 show absolute and changes in plasma PPL activity levels within and between the study arms. Plasma PPL activity levels decreased significantly from baseline to study end for rosuvastatin users (mean change, −0.48 mU/ml; 95% confidence interval [CI] −0.81 to −0.15), while a minor increase was observed for non-users (mean change, 0.17 mU/ml; 95% CI −0.18 to 0.53) for overall VTE. Similar trends were observed in subgroup analyses of participants with a history of provoked and unprovoked VTE, as well as for DVT and PE. However, a pronounced change in plasma PPL activity was observed for the PE patients in the rosuvastatin group (mean difference, −0.94 mU/ml; 95% CI −1.52 to −0.36), and particularly for provoked PE (mean difference, −1.14 mU/ml; 95% CI −2.13 to −0.16; Table 2 and Figure 2). The absolute and relative changes in PPL activity between the two study arms are shown in Table 3. Rosuvastatin treatment yielded a 22% (95% CI −38.2 to −5.8) reduction in PPL activity among all VTEs, and 37% (95% CI −62.9 to −11.2) reduction in PPL activity in participants with a history of PE. The treatment effect of rosuvastatin on PPL activity for overall VTEs, DVTs, and PEs are further illustrated in Figure 3. The treatment effect was also investigated in a linear model adjusted for age and sex, as these parameters were not balanced between groups at baseline.[21] Adjustments for age and sex only marginally altered the mean differences between groups as well as the treatment effects (Table 3).

Total cholesterol levels were reduced from baseline to study end in the rosuvastatin treatment group by 35% (1.96 mmol/L) and by 3% (0.17 mmol/L) in the no treatment group. To explore whether the reduction in PPL activity by statin treatment was explained by

TABLE 1 Baseline characteristics of the study participants included in analysis

	Rosuvastatin treatment ($n = 125$)	No treatment ($n = 120$)
General		
Age (years)	57 (19–83)	59 (21–81)
Male	67 (53.6)	83 (69.2)
BMI (kg/m^2)	27.4 (19.2–43.5)	27.8 (17.2–43.3)
Baseline cholesterol (mmol/L)	5.59 (2.95–8.98)	5.59 (3.33–7.89)
Aspirin use	5 (4)	5 (4.2)
Venous thromboembolism characteristics		
Deep vein thrombosis	71 (56.8)	64 (53.3)
Pulmonary embolism	54 (43.2)	56 (46.7)
Unprovoked	56 (44.8)	63 (52.5)
Provoked	69 (55.2)	57 (47.5)
Surgery/trauma/immobilization	32 (25.6)	31 (25.8)
Travel >4 h	22 (17.6)	14 (11.7)
Estrogen use (% in women)	24 (41.4)	14 (37.8)
Pregnancy/puerperium (% in women)	0 (0)	2 (5.4)
Malignancy	2 (1.6)	8 (6.7)
Recurrent venous thromboembolism	10 (8)	8 (6.7)
Cardiovascular risk factors		
Absent	37 (29.6)	25 (20.8)
Present	88 (70.4)	95 (79.2)
Current smoking	18 (14.4)	17 (14.2)
Hypertension	24 (19.2)	21 (17.5)
Diabetes	3 (2.4)	0 (0)
Overweight (25 ≤ BMI < 30)	53 (42.4)	51 (42.5)
Obese (30 ≤ BMI)	29 (23.2)	35 (29.2)

Note: Continuous variables denoted as mean (range) and categorical variables as number of (%).
Abbreviation: BMI, body mass index.

the statin-dependent decrease in serum cholesterol, we plotted the absolute changes in total cholesterol against changes in PPL activity (Figure 4). A weak and Pearson's correlation coefficient of −0.10 (*P*-value .28) indicates that the reduction in PPL activity by statin treatment was independent of the cholesterol-lowering effect.

To assess whether the observed effect of statin treatment on PPL activity could potentially be explained by alterations in EV count, EVs were isolated from plasma by ultracentrifugation; labeled with lactadherin (which binds to membranes expressing PS); and CD41, a platelet specific marker; and counted using a sensitive flow cytometer. Plasma levels of lactadherin-positive and platelet-derived EV, assessed as percent change from baseline within each treatment group, are shown as box plots in Figure 5. The box plots show a modest percentage increase in total EV (Figure 5, left panel) and platelet-derived EVs (Figure 5, right panel) for the no treatment group, but no difference in effect between treatment groups. The absolute numbers of EVs per μL and mean differences in lactadherin-positive and CD41-positive EV counts are listed in Tables S1 and S2 in supporting information, respectively.

4 | DISCUSSION

In the present study, we investigated the effect of rosuvastatin treatment on plasma PPL activity, measured by a FXa-dependent PPL clotting assay, in patients with a history of VTE. Statin treatment caused a 22% reduction in PPL activity for all VTE patients and 37% reduction in PPL activity for PE patients compared to no treatment. The observed effect of rosuvastatin on PPL activity was not explained by changes in serum levels of total cholesterol or a parallel change in plasma levels of total- and platelet-derived microvesicles by statin treatment. The results from our study support the beneficial effect of statin treatment on coagulation factors and thrombin generation potential in plasma. As the presence of negatively charged phospholipids augment the activity of the extrinsic tenase complex, TF-FVIIa, by several orders of magnitude,[26] the combined effect of reduced PPL activity and modest decline in several coagulation factors may reduce coagulation activation and contribute to explain why rosuvastatin treatment lowers the risk of VTE.[15]

Clinical studies have shown that statin treatment, either with simvastatin,[30] atorvastatin,[31,32] or cerivastatin,[33] caused a beneficial

TABLE 2 Mean difference in measures of PPL activity (mU/ml) from baseline to study end within the treatment and no treatment group and between groups for all VTE and subgroups provoked and unprovoked VTE, DVT, and PE

	Treatment group (T) ($n = 125$)			No treatment group (NT) ($n = 120$)			t-test	Linear model[a]
Subgroup	Baseline	Study end	Change over study	Baseline	Study end	Change over study	Change T – Change NT	Regression coefficient statins
VTE								
All	3.30 ± 2.47	2.82 ± 2.08	−0.48 (−0.81, −0.15)	2.64 ± 1.81	2.82 ± 2.22	0.17 (−0.18, 0.53)	−0.66 (−1.14, −0.17)	−0.63 (−1.12, −0.14)
Provoked	3.32 ± 2.56	2.88 ± 2.24	−0.44 (−0.93, 0.06)	2.62 ± 1.65	2.78 ± 2.31	0.15 (−0.44, 0.75)	−0.59 (−1.35, 0.17)	−0.54 (−1.31, 0.23)
Unprovoked	3.29 ± 2.37	2.75 ± 1.88	−0.54 (−0.97, −0.10)	2.66 ± 1.95	2.85 ± 2.14	0.19 (−0.24, 0.62)	−0.73 (−1.33, −0.12)	−0.72 (−1.33, −0.10)
DVT								
All	2.99 ± 2.10	2.85 ± 2.09	−0.13 (−0.51, 0.24)	2.50 ± 1.64	2.59 ± 1.89	0.09 (−0.31, 0.50)	−0.23 (−0.77, 0.32)	−0.22 (−0.77, 0.32)
Provoked	2.74 ± 1.97	2.85 ± 2.24	0.11 (−0.31, 0.52)	2.76 ± 1.53	2.76 ± 1.69	0.00 (−0.60, 0.60)	0.11 (−0.58, 0.80)	0.13 (−0.57, 0.83)
Unprovoked	3.29 ± 2.24	2.86 ± 1.92	−0.43 (−1.10, 0.24)	2.29 ± 1.72	2.45 ± 2.05	0.16 (−0.41, 0.73)	−0.59 (−1.45, 0.27)	−0.60 (−1.47, 0.27)
PE								
All	3.72 ± 2.85	2.78 ± 2.09	−0.94 (−1.52, −0.36)	2.81 ± 1.98	3.08 ± 2.53	0.27 (−0.35, 0.89)	−1.21 (−2.05, −0.37)	−1.13 (−2.01, −0.26)
Provoked	4.07 ± 3.05	2.92 ± 2.29	−1.14 (−2.13, −0.16)	2.49 ± 1.78	2.79 ± 2.82	0.30 (−0.75, 1.36)	−1.45 (−2.86, −0.04)	−1.34 (−2.85, 0.18)
Unprovoked	3.28 ± 2.58	2.60 ± 1.85	−0.68 (−1.21, −0.15)	3.16 ± 2.16	3.39 ± 2.18	0.23 (−0.45, 0.92)	−0.91 (−1.77, −0.05)	−0.90 (−1.79, −0.00)

Note:: Values are means ± 1 standard deviation (SD) or the mean difference between groups with 95% confidence intervals in parentheses.

Abbreviations: DVT, deep vein thrombosis; PE, pulmonary embolism; PPL, procoagulant phospholipids; VTE, venous thromboembolism.

[a]Adjusted for age and sex.

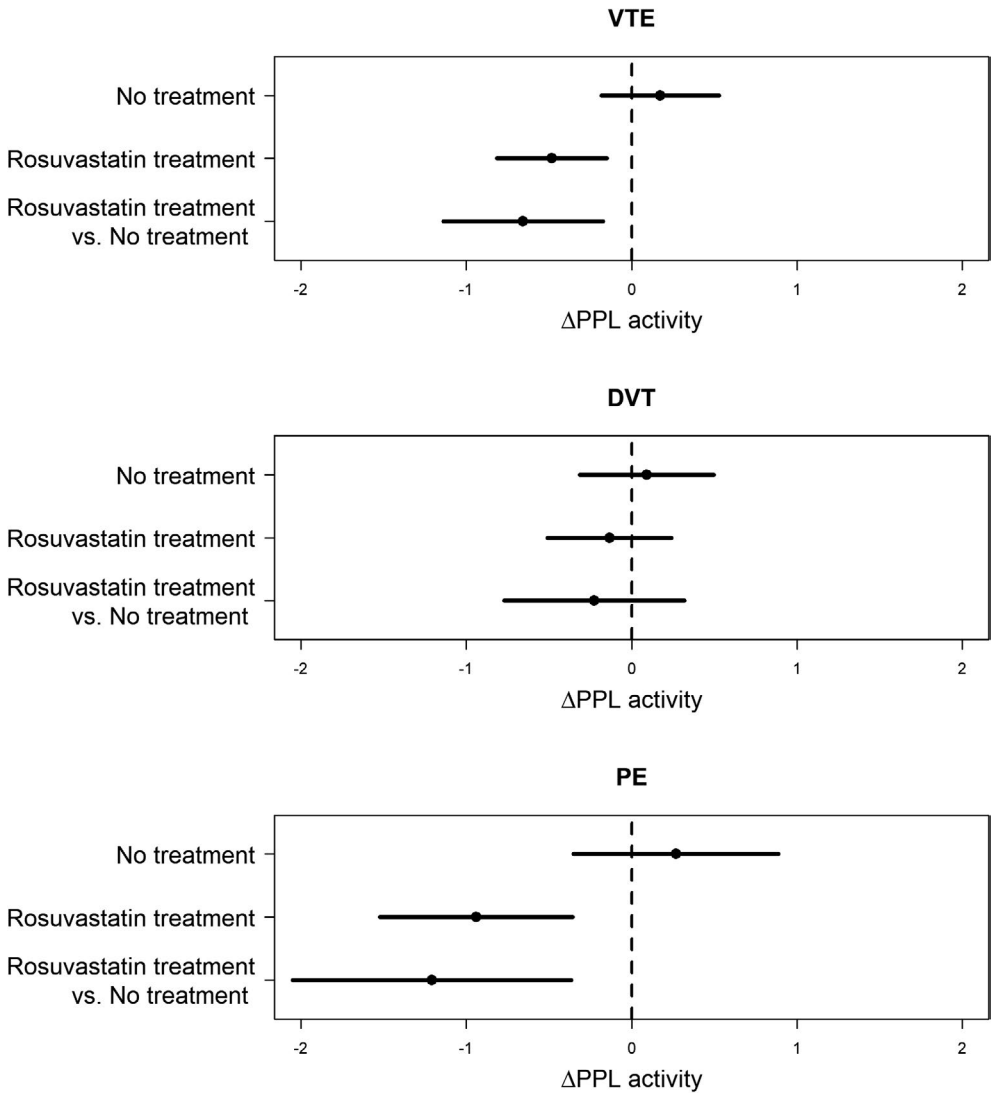

FIGURE 2 Forest plots of changes in plasma procoagulant phospholipid (PPL) activity (mU/ml; after minus before) within the rosuvastatin treatment and the no treatment group and between groups for all venous thromboembolisms (VTEs), deep vein thromboses (DVTs), and pulmonary embolisms (PEs). Values are means with 95% confidence intervals

effect on the coagulation system by a moderate lowering of specific coagulation factors and thrombin generation. In the START trial, rosuvastatin treatment showed favorable effects on the hemostatic system by reducing plasma levels of coagulation factors FVII, FVIII, and FXI by 4–6%,[21] D-dimer by 3%;[21] lowered the *ex vivo* thrombin generation potential by 10%;[22] and increased the fibrinolytic potential assessed by shortening of the mean plasma clot lysis time and a decrease in both plasmin inhibitor levels and thrombin-activatable fibrinolysis inhibitor (TAFI) activity.[23] The treatment effects of rosuvastatin on thrombin generation and plasma D-dimer levels were mainly driven by an increase among non-statin users.[21,22] In contrast, we found a more profound beneficial effect of rosuvastatin treatment that was mainly driven by a significant decline in the PPL activity among rosuvastatin users accompanied by a minor increase in the PPL activity among the non-users. The increase in hemostatic factors among non-statin users in our and previous studies from the START trial may be interpreted as a result of the rebound hypercoagulability often seen after discontinuation of anticoagulant treatment.[34,35]

Previous studies have demonstrated that plasma PPL activity is mainly due to the presence of EVs,[27,28] and most,[36-40] but not all[41] case-control studies have reported increased EV-related plasma PPL activity in VTE patients compared to controls. Therefore, our findings of a profound decrease in PPL activity by statin treatment may contribute to the reduction of incident and recurrent VTE by statin treatment.[12-20] Microvesicles (MVs) are larger EVs (100–1000 nm in diameter), which bud directly from the plasma membrane of activated cells, and express surface markers of their cell of origin.[42,43] The largest proportion of MVs in circulating blood is derived from platelets[44,45] and the subsequent procoagulant activity in plasma is mediated by platelet-derived MVs (PDMVs).[36,44] A strong inverse correlation has also been reported between PPL clotting time and lactadherin-positive EVs measured in PPP from healthy control subjects and patients with obstructive

TABLE 3 Treatment effect of rosuvastatin on measures of PPL activity in percentage change and as a linear model for all VTE and subgroups provoked- and unprovoked VTE, DVT, and PE

Subgroup	Delta T – Delta NT	Percentage change (%)	Linear model adjusted effect[a]	Adjusted percentage effect[a] (%)
VTE				
All	−0.66 (−1.14, −0.17)	−22.0 (−38.2, −5.8)	−0.63 (−1.12, −0.14)	−21.0 (−37.5, −4.6)
Provoked	−0.59 (−1.35, 0.17)	−19.7 (−45.0, 5.6)	−0.22 (−0.77, 0.32)	−18.0 (−43.8, 7.7)
Unprovoked	−0.73 (−1.33, −0.12)	−24.7 (−45.1, −4.2)	−0.72 (−1.33, −0.10)	−24.3 (−45.1, −3.5)
DVT				
All	−0.23 (−0.77, 0.32)	−8.2 (−28.0, 11.6)	−0.22 (−0.77, 0.32)	−8.1 (−27.9, 11.8)
Provoked	0.11 (−0.58, 0.80)	3.9 (−21.2, 29.0)	0.13 (−0.57, 0.83)	4.6 (−20.8, 30.0)
Unprovoked	−0.59 (−1.45, 0.27)	−21.4 (−52.5, 9.7)	−0.60 (−1.47, 0.27)	−21.7 (−53.3, 9.9)
PE				
All	−1.21 (−2.05, −0.37)	−37.0 (−62.9, −11.2)	−1.13 (−2.01, −0.26)	−34.8 (−61.7, −8.0)
Provoked	−1.45 (−2.86, −0.04)	−43.9 (−86.8, −1.1)	−1.34 (−2.85, 0.18)	−40.7 (−86.6, 5.3)
Unprovoked	−0.91 (−1.77, −0.05)	−28.4 (−55.2, −1.6)	−0.90 (−1.79, −0.00)	−27.9 (−55.7, −0.1)

Note:: Values are mean differences between groups with 95% confidence intervals in parentheses or percentage change calculated using the mean difference and dividing it by the mean baseline levels of PPL for both groups.

Abbreviations: DVT, deep vein thrombosis; PE, pulmonary embolism; PPL, procoagulant phospholipids; VTE, venous thromboembolism.

[a]Adjusted for age and sex.

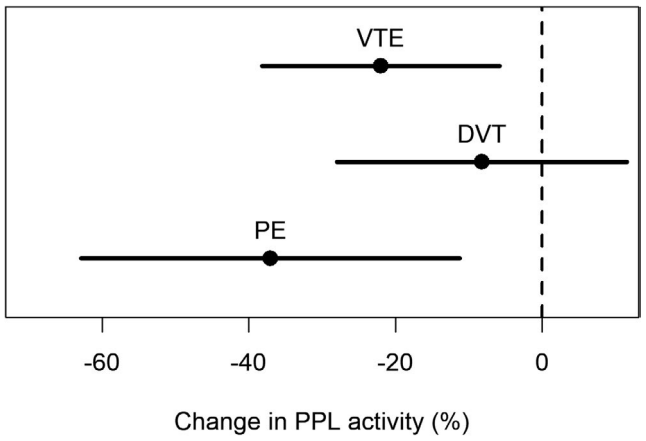

FIGURE 3 Forest plot of the treatment effects (change within the statin group minus the change in the no treatment group) as percentage change in procoagulant phospholipid (PPL) activity for all venous thromboembolisms (VTEs), deep vein thromboses (DVTs), and pulmonary embolisms (PEs). Values are means with 95% confidence intervals

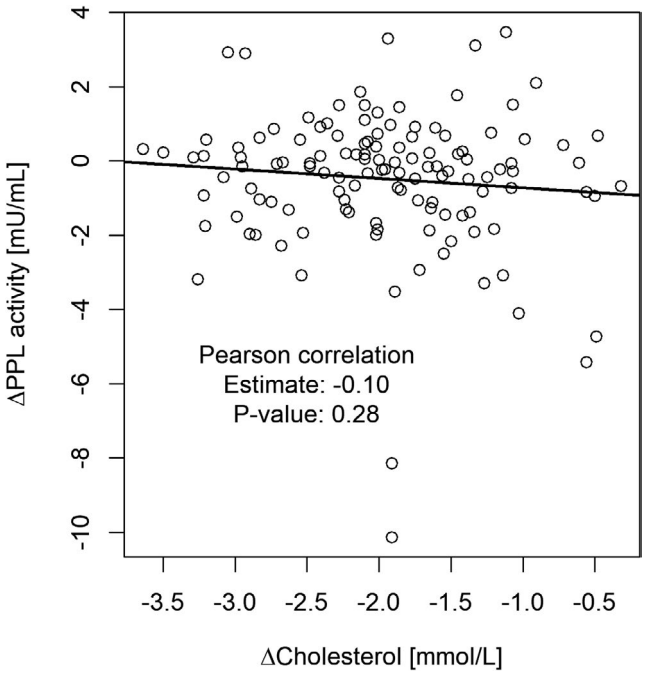

FIGURE 4 Change in individual total cholesterol levels from baseline to study end plotted against change in individual procoagulant phospholipid (PPL) activity for the treatment group

sleep apnoea (OSA), though the strength of the correlations was mainly driven by the OSA patients.[28] We therefore hypothesized that the reduction we observed in plasma PPL activity following rosuvastatin treatment was caused by a parallel decline in plasma MV levels, and particularly platelet-derived MVs. To test our hypothesis, we isolated EVs from PFP and measured the total count (lactadherin-positive) and platelet-derived MVs (lactadherin- and CD41-positive) by flow cytometry. Although we found statin treatment to lower the PPL activity in the treatment group, we did not observe a reduction in total EV count, nor platelet-derived EVs, for comparisons between—or within—study groups.

Our results show that rosuvastatin treatment did not affect plasma MV levels in patients with a history of VTE. Contradicting our findings, previous observational studies have shown that patients with arterial cardiovascular diseases or risk factors (hyperlipidemia in particular) had higher plasma MV levels than control individuals, and that statin treatment lowered plasma MV levels in most, but not all, studies.[46-51] Several factors may contribute to

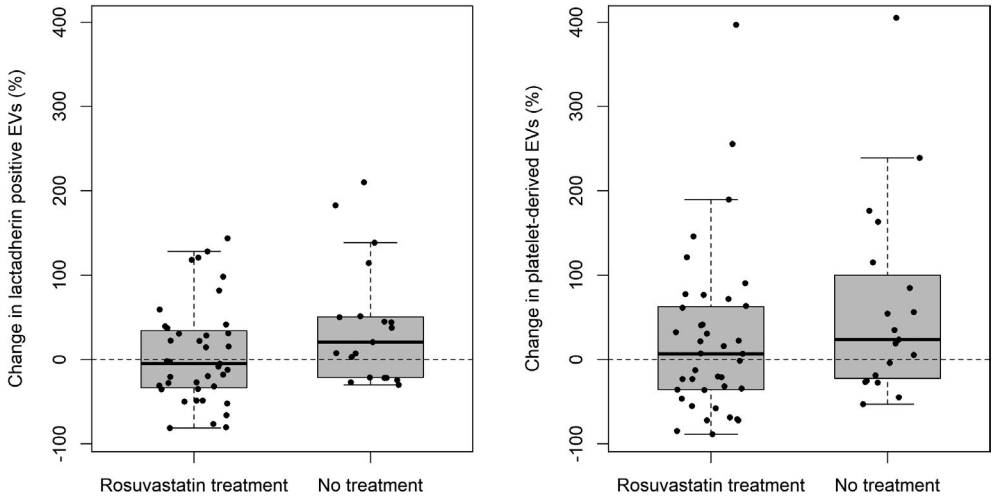

FIGURE 5 The effect of rosuvastatin treatment and no treatment on percentage change of lactadherin-positive (left panel) and platelet-derived (right panel) extracellular vesicles from baseline to study end in plasma measured by flow cytometry

explain our findings. First, the effect of statin treatment on plasma MV levels may be limited to individuals with arterial cardiovascular diseases and risk factors, and not transferrable to VTE patients. Second, one might speculate that statin treatment could differentially influence EV formation from various intravascular cells and the subsequent process of externalization of PS to the outer leaflet of the cell membrane during EV formation.[52] Accordingly, in a placebo-controlled randomized double-blinded crossover study, the treatment of 19 patients with peripheral arterial occlusive disease for 8 weeks with 80 mg atorvastatin daily showed a reduction in plasma MV levels expressing CD62P- and CD61-positive MVs without affecting plasma levels of lactadherin-positive EVs.[49] Third, a well-recognized limitation of flow cytometry as a method is the detection limit of the instrument. Even a sensitive flow cytometer will still only detect vesicles above approximately 200 nm in diameter, and thereby exclude the smaller-sized population of EVs. Vesicles larger than 200 nm in diameter have been reported to only account for a minority of the EV population (<5%). This may imply that a possible decrease in plasma EVs after statin treatment could have been masked by the unchanged level of EVs >200 nm in diameter.[53]

Some aspects of our randomized controlled trial need attention. Neither the patients nor the physicians were blinded to treatment. However, it is unlikely that knowledge of the treatment would affect the laboratory outcomes. Furthermore, the technicians conducting the laboratory analyses were blinded to sample treatment. In addition, despite randomization, the distribution of age and sex was uneven between the study arms. We decided a priori to adjust analysis for age and sex as potential confounders, and adjustments did not influence the observed treatment effect. Even though results from subgroup analysis revealed the most pronounced decrease in plasma PPL activity in individuals with a history of PE, they should be interpreted with caution as the study was not originally powered to analyze differences in subgroups.[21] Last, as participants were recruited from an outpatient setting, it limits the risk of confounding diseases at randomization, and for analysis, participants were compared with themselves. Unfortunately, it was not possible to assess whether anti-inflammatory effects of statin treatment could partly explain the beneficial effect of statins on plasma PPL or modify the statin effect as plasma CRP was not measured in the START trial.

In conclusion, rosuvastatin treatment caused a substantial decrease in plasma PPL activity, suggesting that PPL-dependent attenuation of coagulation activation may contribute to a reduced VTE risk by statin treatment. Further studies are warranted to validate our findings and unravel underlying mechanisms.

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CONFLICTS OF INTEREST

The authors state that they have no conflicts of interest.

AUTHOR CONTRIBUTIONS

C. Ramberg planned experiments, analyzed data, and wrote and revised the manuscript. K. Hindberg performed the statistical analysis and revised the manuscript. O. Snir planned experiments, analyzed data, and participated in writing and revising the manuscript. J. S. Biederman, S. C. Cannegieter, F. J. van der Meer, F. W. G. Leebeek, M. J. H. A. Kruip, and W. M. Lijfering revised the manuscript. W. M. Lijfering was responsible for the START study concept. J. B. Hansen conceived and designed the study, analyzed data, and participated in writing and revising the manuscript.

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REFERENCES

1. White RH. The epidemiology of venous thromboembolism. *Circulation*. 2003;107:4-8.
2. Heit JA, Spencer FA, White RH. The epidemiology of venous thromboembolism. *J Thromb Thrombolysis*. 2016;41:3-14.
3. Wolberg AS, Rosendaal FR, Weitz JI, et al. Venous thrombosis. *Nat Rev Dis Primers*. 2015;1:15006.
4. Kearon C, Akl EA, Ornelas J, et al. Antithrombotic therapy for VTE disease: CHEST guideline and expert panel report. *Chest*. 2016;149:315-352.
5. Beyer-Westendorf J, Forster K, Pannach S, et al. Rates, management, and outcome of rivaroxaban bleeding in daily care: results from the Dresden NOAC registry. *Blood*. 2014;124:955-962.
6. Ost D, Tepper J, Mihara H, Lander O, Heinzer R, Fein A. Duration of anticoagulation following venous thromboembolism: a meta-analysis. *JAMA*. 2005;294:706-715.
7. Veeger NJ, Piersma-Wichers M, Tijssen JG, Hillege HL, van der Meer J. Individual time within target range in patients treated with vitamin K antagonists: main determinant of quality of anticoagulation and predictor of clinical outcome. A retrospective study of 2300 consecutive patients with venous thromboembolism. *Br J Haematol*. 2005;128:513-519.
8. Schulman S, Lindmarker P, Holmstrom M, et al. Post-thrombotic syndrome, recurrence, and death 10 years after the first episode of venous thromboembolism treated with warfarin for 6 weeks or 6 months. *J Thromb Haemost*. 2006;4:734-742.
9. Prandoni P, Lensing AW, Cogo A, et al. The long-term clinical course of acute deep venous thrombosis. *Ann Intern Med*. 1996;125:1-7.
10. Heit JA, Mohr DN, Silverstein MD, Petterson TM, O'Fallon WM, Melton LJ 3rd. Predictors of recurrence after deep vein thrombosis and pulmonary embolism: a population-based cohort study. *Arch Intern Med*. 2000;160:761-768.
11. Prandoni P, Noventa F, Ghirarduzzi A, et al. The risk of recurrent venous thromboembolism after discontinuing anticoagulation in patients with acute proximal deep vein thrombosis or pulmonary embolism. A prospective cohort study in 1,626 patients. *Haematologica*. 2007;92:199-205.
12. Kunutsor SK, Seidu S, Khunti K. Statins and primary prevention of venous thromboembolism: a systematic review and meta-analysis. *Lancet Haematol*. 2017;4:e83-e93.
13. Pai M, Evans NS, Shah SJ, Green D, Cook D, Crowther MA. Statins in the prevention of venous thromboembolism: a meta-analysis of observational studies. *Thromb Res*. 2011;128:422-430.
14. Hippisley-Cox J, Coupland C. Unintended effects of statins in men and women in England and Wales: population based cohort study using the QResearch database. *BMJ*. 2010;340:c2197.
15. Glynn RJ, Danielson E, Fonseca FA, et al. A randomized trial of rosuvastatin in the prevention of venous thromboembolism. *N Engl J Med*. 2009;360:1851-1861.
16. Rahimi K, Bhala N, Kamphuisen P, et al. Effect of statins on venous thromboembolic events: a meta-analysis of published and unpublished evidence from randomised controlled trials. *PLoS Med*. 2012;9:e1001310.
17. Kunutsor SK, Seidu S, Khunti K. Statins and secondary prevention of venous thromboembolism: pooled analysis of published observational cohort studies. *Eur Heart J*. 2017;38:1608-1612.
18. Biere-Rafi S, Hutten BA, Squizzato A, et al. Statin treatment and the risk of recurrent pulmonary embolism. *Eur Heart J*. 2013;34:1800-1806.
19. Schmidt M, Cannegieter SC, Johannesdottir SA, Dekkers OM, Horvath-Puho E, Sorensen HT. Statin use and venous thromboembolism recurrence: a combined nationwide cohort and nested case-control study. *J Thromb Haemost*. 2014;12:1207-1215.
20. Smith NL, Harrington LB, Blondon M, et al. The association of statin therapy with the risk of recurrent venous thrombosis. *J Thromb Haemost*. 2016;14:1384-1392.
21. Biedermann JS, Kruip M, van der Meer FJ, et al. Rosuvastatin use improves measures of coagulation in patients with venous thrombosis. *Eur Heart J*. 2018;39:1740-1747.
22. Orsi FA, Biedermann JS, Kruip M, et al. Rosuvastatin use reduces thrombin generation potential in patients with venous thromboembolism: a randomized controlled trial. *J Thromb Haemost*. 2019;17:319-328.
23. Schol-Gelok S, de Maat MPM, Biedermann JS, et al. Rosuvastatin use increases plasma fibrinolytic potential: a randomised clinical trial. *Br J Haematol*. 2020;190:916-922.
24. Biedermann JS, Cannegieter SC, Roest M, et al. Platelet reactivity in patients with venous thrombosis who use rosuvastatin: a randomized controlled clinical trial. *J Thromb Haemost*. 2016;14:1404-1409.
25. Zwaal RF, Comfurius P, Bevers EM. Lipid-protein interactions in blood coagulation. *Biochim Biophys Acta*. 1998;1376:433-453.
26. Ruf W, Rehemtulla A, Morrissey JH, Edgington TS. Phospholipid-independent and -dependent interactions required for tissue factor receptor and cofactor function. *J Biol Chem*. 1991;266:2158-2166.
27. Connor DE, Exner T, Ma DD, Joseph JE. Detection of the procoagulant activity of microparticle-associated phosphatidylserine using XACT. *Blood Coagul Fibrinolysis*. 2009;20:558-564.
28. Ayers L, Harrison P, Kohler M, Ferry B. Procoagulant and platelet-derived microvesicle absolute counts determined by flow cytometry correlates with a measurement of their functional capacity. *J Extracell Vesicles*. 2014;3:25348.
29. Ramberg C, Jamaly S, Latysheva N, et al. A modified clot-based assay to measure negatively charged procoagulant phospholipids. *Sci Rep*. 2021;11:9341.
30. Szczeklik A, Musial J, Undas A, et al. Inhibition of thrombin generation by simvastatin and lack of additive effects of aspirin in patients with marked hypercholesterolemia. *J Am Coll Cardiol*. 1999;33:1286-1293.
31. Cortellaro M, Cofrancesco E, Arbustini E, et al. Atorvastatin and thrombogenicity of the carotid atherosclerotic plaque: the ATROCAP study. *Thromb Haemost*. 2002;88:41-47.
32. Macchia A, Laffaye N, Comignani PD, et al. Statins but not aspirin reduce thrombotic risk assessed by thrombin generation in diabetic patients without cardiovascular events: the RATIONAL trial. *PLoS One*. 2012;7:e32894.
33. Ural AU, Yilmaz MI, Avcu F, Yalcin A. Treatment with cerivastatin in primary mixed hyperlipidemia induces changes in platelet aggregation and coagulation system components. *Int J Hematol*. 2002;76:279-283.
34. Martinez C, Katholing A, Folkerts K, Cohen AT. Risk of recurrent venous thromboembolism after discontinuation of vitamin K antagonist treatment: a nested case-control study. *J Thromb Haemost*. 2016;14:1374-1383.
35. Palareti G, Legnani C, Guazzaloca G, et al. Activation of blood coagulation after abrupt or stepwise withdrawal of oral anticoagulants–a prospective study. *Thromb Haemost*. 1994;72:222-226.
36. Bal L, Ederhy S, Di Angelantonio E, et al. Circulating procoagulant microparticles in acute pulmonary embolism: a case-control study. *Int J Cardiol*. 2010;145:321-322.
37. Owen BA, Xue A, Heit JA, Owen WG. Procoagulant activity, but not number, of microparticles increases with age and in individuals after a single venous thromboembolism. *Thromb Res*. 2011;127:39-46.
38. Campello E, Spiezia L, Radu CM, et al. Circulating microparticles in carriers of prothrombin G20210A mutation. *Thromb Haemost*. 2014;112:432-437.
39. Campello E, Spiezia L, Radu CM, et al. Circulating microparticles and the risk of thrombosis in inherited deficiencies of antithrombin, protein C and protein S. *Thromb Haemost*. 2016;115:81-88.

40. Campello E, Spiezia L, Radu CM, et al. Circulating microparticles in carriers of factor V Leiden with and without a history of venous thrombosis. *Thromb Haemost*. 2012;108:633-639.
41. Ay C, Freyssinet JM, Sailer T, Vormittag R, Pabinger I. Circulating procoagulant microparticles in patients with venous thromboembolism. *Thromb Res*. 2009;123:724-726.
42. Owens AP 3rd, Mackman N. Microparticles in hemostasis and thrombosis. *Circ Res*. 2011;108:1284-1297.
43. Zara M, Guidetti GF, Camera M, et al. Biology and role of extracellular vesicles (EVs) in the pathogenesis of thrombosis. *Int J Mol Sci*. 2019;20:2840.
44. Berckmans RJ, Nieuwland R, Boing AN, Romijn FP, Hack CE, Sturk A. Cell-derived microparticles circulate in healthy humans and support low grade thrombin generation. *Thromb Haemost*. 2001;85:639-646.
45. Aatonen M, Gronholm M, Siljander PR. Platelet-derived microvesicles: multitalented participants in intercellular communication. *Semin Thromb Hemost*. 2012;38:102-113.
46. Suades R, Padro T, Alonso R, Mata P, Badimon L. Lipid-lowering therapy with statins reduces microparticle shedding from endothelium, platelets and inflammatory cells. *Thromb Haemost*. 2013;110:366-377.
47. Pawelczyk M, Chmielewski H, Kaczorowska B, Przybyla M, Baj Z. The influence of statin therapy on platelet activity markers in hyperlipidemic patients after ischemic stroke. *Arch Med Sci*. 2015;11:115-121.
48. Nomura S, Shouzu A, Omoto S, Nishikawa M, Fukuhara S, Iwasaka T. Losartan and simvastatin inhibit platelet activation in hypertensive patients. *J Thromb Thrombolysis*. 2004;18:177-185.
49. Mobarrez F, He S, Broijersen A, et al. Atorvastatin reduces thrombin generation and expression of tissue factor, P-selectin and GPIIIa on platelet-derived microparticles in patients with peripheral arterial occlusive disease. *Thromb Haemost*. 2011;106:344-352.
50. Pinheiro LF, Franca CN, Izar MC, et al. Pharmacokinetic interactions between clopidogrel and rosuvastatin: effects on vascular protection in subjects with coronary heart disease. *Int J Cardiol*. 2012;158:125-129.
51. Sommeijer DW, Joop K, Leyte A, Reitsma PH, ten Cate H. Pravastatin reduces fibrinogen receptor gpIIIa on platelet-derived microparticles in patients with type 2 diabetes. *J Thromb Haemost*. 2005;3:1168-1171.
52. Rosinska J, Lukasik M, Kozubski W. The impact of vascular disease treatment on platelet-derived microvesicles. *Cardiovasc Drugs Ther*. 2017;31:627-644.
53. Jamaly S, Ramberg C, Olsen R, et al. Impact of preanalytical conditions on plasma concentration and size distribution of extracellular vesicles using nanoparticle tracking analysis. *Sci Rep*. 2018;8:17216.

SUPPORTING INFORMATION

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