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LDL extracellular vesicle coagulation protein levels change after initiation of statin therapy. Findings from the METEOR trial

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ABSTRACT

Background: Statins are thought to have pleiotropic properties, including anticoagulant effects, in addition to reducing lipoprotein (LDL) levels. Plasma extracellular vesicles (EVs) are small bilayer membrane vesicles involved in various biological processes including coagulation. Since subsets of EVs in the LDL plasma fraction (LDL-EVs) correlate with thrombin activity, we hypothesized that changes in LDL-EVs after statin therapy may differ from that of serum levels of coagulation proteins, providing insight into the effects of statins on coagulation.

Methods: The study was conducted in 666 subjects with available serum from the METEOR trial, a trial of the effect of rosuvastatin versus placebo in patients with subclinical atherosclerosis. Changes in protein levels of von Willebrand Factor (VWF), SerpinC1 and plasminogen were measured in serum and in LDL-EVs, and were compared between the rosuvastatin and placebo groups.

Results: LDL-EV levels of plasminogen and VWF increased with rosuvastatin treatment compared to placebo (mean change of 126 ± 8 versus 17 ± 12 $\mu\text{g/mL}$ for plasminogen ($p < 0.001$) and 310 ± 60 versus 64 ± 55 $\mu\text{g/mL}$ for VWF ($p = 0.015$)). There was no difference between groups for change in LDL-EV-SerpinC1. In contrast, serum plasminogen levels increased to a lesser extent with rosuvastatin compared to placebo (23 ± 29 versus 67 ± 17 $\mu\text{g/mL}$, $p = 0.024$) and serum VWF levels showed no significant difference between both groups.

Conclusions: Rosuvastatin increases LDL-EV coagulation proteins plasminogen and VWF in patients with subclinical atherosclerosis, an effect that is different from the effect of rosuvastatin on the same proteins in serum. This identifies LDL-EVs as a newly detected possible intermediate between statin therapy and coagulation.

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1. Introduction

Lipoproteins transport water-insoluble lipids through the blood. High levels of low-density lipoprotein (LDL) are an established risk factor of cardiovascular disease (CVD) [1,2]. Statin treatment significantly

reduces LDL levels and reduces CVD risk [3,4], but the mechanism through which statins prevent cardiovascular disease may not be entirely due to LDL reduction [5]. Statins may have pleiotropic effects such as on regression of atherosclerosis [6], stabilization of plaque [7] and reduction of inflammation [7], all of which contribute to a reduced risk of cardiovascular events [3,4].

Furthermore, statins seem to beneficially affect the anticoagulant profile as well [8], resulting in a fairly acute reduction in thrombotic events [9]. Lowering of LDL through LDL apheresis has been shown to result in an acute reduction of coagulation proteins in plasma [10]. However, the mechanisms through which statin lipid-lowering therapies affect coagulation remain to be elucidated.

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Plasma extracellular vesicles (EVs) are small bilayer membrane vesicles abundantly present in plasma which are important in the cell-to-cell communication in a variety of biological processes including inflammation and coagulation [11,12]. EVs can be produced by all cell types and contain surface molecules from their parent cells, as well as selected cytosolic content (proteins, lipids, RNA, microRNA). EVs are distinct from lipid particles which contain a monolayer membrane and mainly consist of lipids and proteins involved in lipid transport. EVs contain various inflammatory and coagulation proteins and can facilitate the assembly of the coagulation cascade via their bilayer membrane [13–15]. Release of EVs increases under inflammatory and hypercoagulable conditions with higher numbers of circulating EVs being associated with the presence of various CVDs including coronary artery disease, subclinical atherosclerosis and thrombosis [12,13]. Furthermore, EVs enriched with coagulation proteins have been associated with future (recurrent) cardiovascular events in CVD patients [12,16]. Recently, a subset of EVs in the LDL plasma fraction (LDL-EVs) was reported to be correlated to thrombin activity [17].

Since statin treatment lowers LDL levels and subsets of LDL-EVs correlate with thrombin activity, we hypothesized that LDL-EVs are involved in the anticoagulant effects of statins. Therefore, changes in coagulation proteins in LDL associated EVs after statin therapy may differ from changes in coagulation proteins in serum.

To test our hypothesis, we measured the levels of 3 coagulation proteins in LDL-EVs and in serum before and after initiation of rosuvastatin therapy. The 3 proteins consisted of a procoagulant protein (von Willebrand factor (VWF)), an anticoagulant protein (SerpinC1) and a fibrinolytic protein (plasminogen). The selection of these three proteins was based on the availability of antibodies and recombinant proteins needed for the assay. These proteins were measured in samples of participants in the Measuring Effects on Intima-Media Thickness: an Evaluation of Rosuvastatin (METEOR) study, a double-blind randomized trial investigating the role of rosuvastatin versus placebo on atherosclerosis progression in an asymptomatic low CVD risk population recruited from North America and Europe [18].

2. Material and methods

2.1. Study population

The present analysis is a sub-study of the METEOR study, which has been published previously [18]. Briefly, METEOR was designed as a randomized, double-blinded trial to investigate whether the administration of 40 mg rosuvastatin daily could reduce atherosclerosis progression, as measured with repeated carotid ultrasound examinations, in an asymptomatic population (no history of CVD and no diabetes) with subclinical atherosclerosis [19]. The METEOR study was conducted in accordance with the ethical principles of the Declaration of Helsinki and the study protocol was approved by the appropriate institutional review board or independent ethics committee at each site. All participants provided written informed consent.

Subjects in the METEOR study were examined at various time points after inclusion. The last visit before randomization, is referred to as time point 1 (T1) in the current study; the final visit of this trial, two years (104 weeks) after T1, is referred to as time point 2 (T2) in the current study. For the purpose of the present study, LDL-EVs were isolated from stored serum samples at T1 and T2.

In total, 984 subjects were recruited in the study; serum samples were obtained from 846 subjects (there was a 5:2 randomization design such that 606 of them received rosuvastatin and 240 of them received placebo). At T2, 496 subjects in rosuvastatin group and 199 subjects in placebo group had follow-up LDL levels. Of those, 477 subjects in rosuvastatin group and 189 subjects in placebo group had reliable protein concentrations in the LDL-EV fraction. These 666 subjects were included in this current study. The selection process of the current study population is depicted in supplementary Fig. S1.

2.2. LDL-EV isolation

Co-precipitation of EVs and LDL was adapted from the well-established method of LDL precipitation [20]. In brief, 6.5% dextran sulphate and 2 M MnCl_2 stocks were prepared, respectively. The dextran sulphate stock (1:125, v/v) and MnCl_2 stock (1:40, v/v) were sequentially added into 125 μL serum. The samples were mixed thoroughly and spun at 4800 g for 10 min at 4 °C. The pellets (LDL-EV fractions) were lysed in 125 μL lysis buffer. All lysates were stored at -80°C until analysis.

2.3. Quantitative protein assay

Quantitative measurement using a beads-based multiplex-immunoassay was performed as described before [16]. In short, the beads (Luminex \# MagPlex-C Microspheres, MC100) were coupled with specific antibodies, and then samples were incubated with the bead-capture antibody complex and the corresponding biotinylated antibodies for detection. Streptavidin-phycoerythrin (SA-PE, BD bioscience \# 554061) was added to bind biotin and to indicate the amount of the target protein. The concentration of the protein was calculated in accordance with respectively corresponding standard curves of homologous recombinant proteins. The Bio-Plex \# 200 Systems (Bio-Rad \# 171-000201) were used for measurement and data analysis. The antibodies and recombinant proteins used in this assay were listed as follows. For VWF: recombinant human VWF protein (Factor VIII free, Fitzgerald \# 30C-CP4003U), anti-human VWF (Fitzgerald \# 70R-10,589) and biotinylated anti-human VWF (Fitzgerald \# 60R-1019); for SerpinC1: antithrombin III antibody (NOVUS \# NBP1-05149), human SerpinC1 biotinylated affinity purified Pab (R&D \# BAF1267), recombinant human SerpinC1 (R&D System \# 1267-PI-010); for plasminogen: anti-human plasminogen (HyTest \# 4P11), biotinylated anti-human plasminogen (HyTest \# 4P11B), recombinant human plasminogen (BBI Solutions \# P204-1).

2.4. Data analysis

Primary endpoints were absolute changes in EV protein levels of VWF, plasminogen and SerpinC1 in the LDL-EV fraction between time point 1 (randomization) and 2 (two-year follow-up) (T2-T1). Secondary endpoints were absolute changes in serum protein levels of VWF, plasminogen and SerpinC1 (T2-T1).

Both the primary endpoints and secondary endpoints were compared between the rosuvastatin and placebo groups. METEOR is a randomized controlled trial, but we discovered some small differences in race and gender between the patients with complete follow-up (e.g. those with reliable EV protein concentrations at T2) and those who were lost to follow-up (see Supplementary data Table S1). Therefore, not only univariable but also multivariable linear regression analyses were performed to assess the relation between rosuvastatin treatment and LDL-EV protein changes. When assumptions of linear regression were violated, quantile regression was used. Potential confounders included in the multivariable analyses were age, gender, body mass index, smoking status and hypertension.

In order to examine whether the effect of rosuvastatin treatment was associated with LDL-reduction, we also analyzed the relation between changes in protein levels and LDL-reduction using linear regression models (or quantile regression when assumptions of linear regression were violated). The reduction of LDL was defined as the difference between the LDL levels at T1 and T2 (e.g. if the difference (T2-T1) is -40 mg/dL , the LDL reduction is 40 mg/dL). The regression models used in LDL reduction analyses were developed in three steps. First only LDL reduction was included as the explanatory variable (univariable analysis); then, the aforementioned potential confounders (age, gender, body mass index, smoking status and hypertension) were added to the model. Finally, the model was additionally adjusted for rosuvastatin treatment. This was done to investigate the impact of rosuvastatin treatment on the regression coefficients of LDL reduction. As an additional analysis, the univariable and multivariable models were applied in the rosuvastatin group and placebo group separately.

All statistical analyses were performed in Rstudio using R software for statistical computing version 3.3.3 [21]. Throughout the analyses a level of significance of 0.05 was used.

3. Results

3.1. Subject characteristics

Baseline (T1) patient characteristics are shown in Table 1. The METEOR cohort consisted of 61% men with mean age of 57 years. They were predominantly Caucasian and slightly obese (median body mass index was 27). The lipid profile of rosuvastatin users improved significantly over the follow-up of 2 years, while among placebo users it did not (Table 1).

3.2. Coagulation protein levels in the LDL-EV fraction in rosuvastatin and placebo treated patients

Rosuvastatin treatment was strongly associated with increase of plasminogen levels in LDL-EVs after two years of treatment (mean change of 126 ± 8 for rosuvastatin versus $17 \pm 12\text{ }\mu\text{g/mL}$ for placebo; $p < 0.001$ for both univariable and multivariable analyses, Fig. 1A and Table 2A). The LDL-EV-VWF levels were also significantly higher in the rosuvastatin group compared to the placebo group after two years (mean change 310 ± 60 versus $64 \pm 55\text{ }\mu\text{g/mL}$; $p = 0.015$ and $p = 0.021$ for univariable and multivariable analyses respectively, Fig. 1A and Table 2A). LDL-EV-SerpinC1 levels showed no difference between two groups. Full multivariable linear regression models (including the

Table 1
Baseline characteristics.

	Placebo <i>n</i> = 189	Rosuvastatin <i>n</i> = 477
Patient characteristics		
Age (years, mean (sd))	57.4 (6.0)	57.5 (6.2)
Male (%)	121 (64.0)	287 (60.2)
White race (%)	185 (97.9)	463 (97.1)
Body mass index (kg/m ² , median [IQR])	27.2 [24.9, 29.7]	26.3 [24.3, 29.1]
Current smoker (%)	7 (3.7)	13 (2.7)
Current alcohol consumer (%)	128 (67.7)	313 (65.6)
Hypertension (%)	42 (22.2)	92 (19.3)
Mean of maximum CIMT (mm, mean (sd))	1.16 (0.20)	1.15 (0.18)
Family history of premature CHD ^a (%)	20 (10.6)	42 (8.8)
C-reactive protein (mg/dL, median [IQR])	0.14 [0.07, 0.28]	0.13 [0.07, 0.28]
eGFR (ml/min per 1.73 m ² , median [IQR])	84.8 [75.7, 93.5]	86.9 [76.5, 95.7]
Lipid and apolipoprotein levels at baseline (T1)		
Total cholesterol (mg/dL, mean (sd))	230.94 (26.9)	229.27 (29.0)
HDL cholesterol (mg/dL, mean (sd))	49.1 (9.2)	49.65 (8.9)
LDL cholesterol (mg/dL, mean (sd))	154.91 (23.7)	154.98 (24.0)
Triglycerides (mg/dL, median [IQR])	120.4 [86.7160.2]	108.8 [79.7149.6]
Apolipoprotein B-100 (mg/dL, mean (sd))	117.46 (17.1)	115.58 (18.2)
Apolipoprotein A-I (mg/dL, mean (sd))	151.72 (19.1)	152.62 (19.9)
Lipid and apolipoprotein changes after 2 years (T2-T1)		
Total cholesterol (mg/dL, mean (SEM))	0.49 (2.0)	−78.90 (1.9)
HDL cholesterol (mg/dL, mean (SEM))	1.45 (0.5)	4.62 (0.4)
LDL cholesterol (mg/dL, mean (SEM))	−1.17 (1.8)	−77.45 (1.7)
Triglycerides (mg/dL, mean (SEM))	1.48 (4.4)	−30.03 (2.5)
Apolipoprotein B-100 (mg/dL, mean (SEM))	−2.38 (1.2)	−48.98 (1.0)
Apolipoprotein A-I (mg/dL, mean (SEM))	5.53 (1.2)	10.41 (0.9)

Abbreviations: SD: Standard deviation; IQR: Interquartile range; CIMT: Carotid intima-media thickness; CHD: Coronary heart disease; eGFR: Estimated glomerular filtration rate; T1: Time point 1 (baseline); T2: Time point 2 (2 years after randomization); HDL: High-density lipoprotein; LDL: Low-density lipoprotein; SEM: Standard error of the mean

^a CHD in a first-degree male relative <55-year-old or first-degree female relative <65-year-old.

estimates for the confounders) of the association between rosuvastatin treatment and LDL-EV coagulation protein changes can be found in Supplementary data Table S2.

3.3. The relation between LDL reduction and changes in LDL-EV coagulation proteins

The change of LDL-EV-plasminogen level was associated with LDL reduction ($\beta = 1.32$, 95% CI = 1.04–1.59): the more LDL reduction, the higher the LDL-EV-plasminogen. This relation remained significant after correction for confounders ($\beta = 1.34$, 95% CI = 1.06–1.62). This relation also appeared to be independent of rosuvastatin treatment since the beta-coefficient did not change much after additional correction for rosuvastatin ($\beta = 1.24$) and the association was observed in the rosuvastatin group and placebo group separately as well (Table 3A).

For both univariable analysis and after adjustment there was no statistically significant association between LDL reduction and LDL-EV levels of VWF or SerpinC1. The results from these regression analyses are shown in Table 3A.

3.4. Serum coagulation proteins levels in rosuvastatin and placebo treated patients

Serum levels of SerpinC1 decreased significantly more in the rosuvastatin group compared to the placebo group after two years of treatment in univariable and multivariable linear regression analyses ($p = 0.032$ and $p = 0.028$ respectively). Changes in serum levels of VWF showed no significant difference between rosuvastatin group and placebo group. Serum plasminogen levels were analyzed with quantile regression analyses, because assumptions of linear regression were not met. This showed that plasminogen levels increased significantly less in the rosuvastatin group than in placebo group ($p = 0.024$ in univariable and $p = 0.018$ in multivariable analyses). These results are summarized in Fig. 1B and Table 2B.

3.5. The relation between LDL reduction and changes in serum coagulation proteins

SerpinC1-serum levels were significantly associated with LDL reduction ($\beta = -2.95$, 95% CI = -4.91 to -0.99). This inverse relationship, with more LDL reduction correlating with lower SerpinC1-serum levels, remained significant after correction for confounders and rosuvastatin treatment. A similar inverse relationship was found in rosuvastatin patients ($\beta = -3.35$, 95% CI = -6.30 to -0.40). In the smaller placebo group this inverse relationship was not significant (Table 3B). No significant relation was found between LDL reduction and serum levels of VWF or plasminogen (Table 3B).

VWF, plasminogen and SerpinC1 levels in LDL-EVs account for an estimated ~13%, ~33% and ~2% respectively, of their total serum levels (including LDL-EV protein; data not shown).

4. Discussion

In the current study, we investigated the effect of rosuvastatin on coagulation protein levels in LDL associated EVs in asymptomatic patients with subclinical atherosclerosis. Compared to placebo, rosuvastatin treatment was strongly associated with an increase in plasminogen and VWF in LDL-EVs, while this association was different for serum values of plasminogen and absent for serum VWF. For plasminogen this effect is probably caused by the LDL reduction, as LDL reduction itself was independently associated with an increase in LDL-EV-plasminogen levels.

To our knowledge, this is the first study that shows an association of statin therapy with coagulation proteins in LDL-EVs identifying LDL-EVs as an intermediate between statin therapy and coagulation.

4.1. Plasminogen

Plasminogen is the precursor of plasmin and a key protein in fibrinolysis [22]. High levels of plasminogen may therefore contribute to

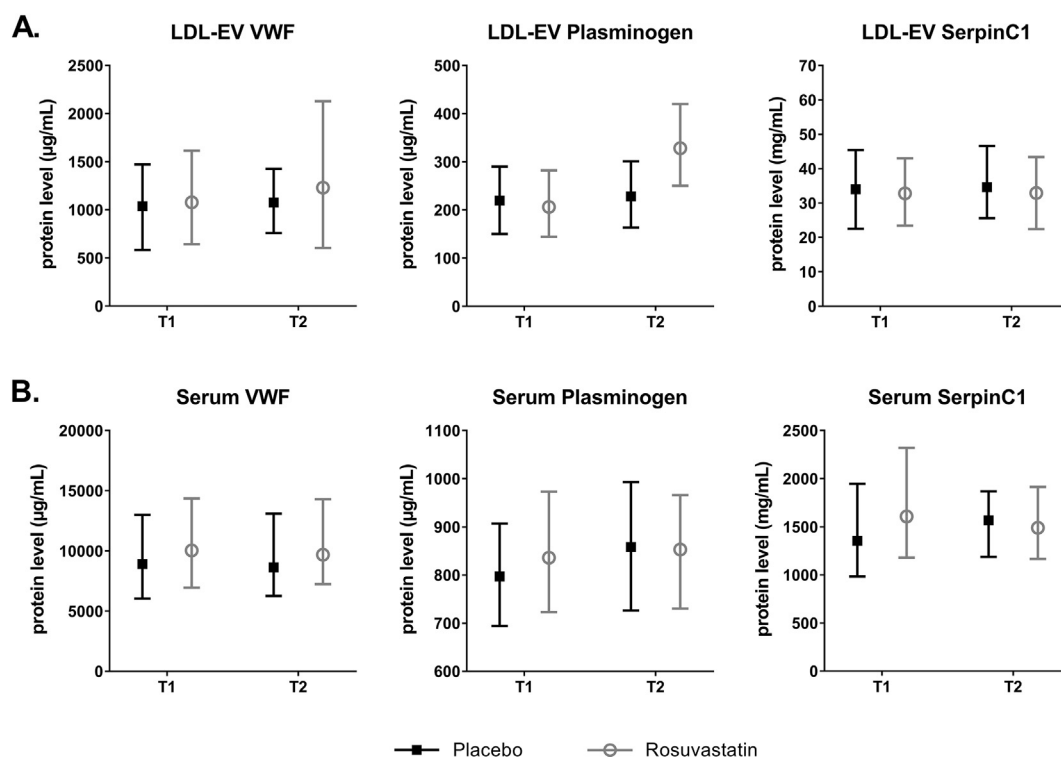


Fig. 1. Median LDL-EVs and serum protein levels before and after two year placebo or rosuvastatin therapy (A) LDL-EV coagulation protein and (B) serum coagulation protein levels before and after two year placebo or rosuvastatin therapy. Protein levels of VWF and plasminogen (in $\mu\text{g/mL}$ serum) and SerpinC1 (in mg/mL serum) for T1 and T2 are shown as median with interquartile range. Exact numbers of protein levels can be found in supplementary Table S3. Abbreviations: LDL: low-density lipoprotein; EV: extracellular vesicle; T1, time point 1; T2, time point 2; VWF: von Willebrand factor.

reduced coagulation. Higher levels of plasminogen activator inhibitor, reducing active plasminogen, have indeed been shown to be a risk factor for CVD [23].

Our study shows that rosuvastatin treatment was strongly associated with an increase in LDL-EV-plasminogen. These results are in line with previously published reports which show that statins enhance tissue plasminogen activator synthesis and reduce plasminogen activator inhibitor synthesis, resulting in a net effect of enhanced fibrinolysis [8,24,25].

Table 2
Changes in LDL-EV and serum coagulation proteins after placebo or rosuvastatin treatment.

	Absolute change		p-Value*	
	Placebo	Rosuvastatin	Univariable	Multivariable
A. LDL-EV				
VWF	64 (55)	310 (60)	0.015	0.021
Plasminogen	17 (12)	126 (8)	<0.001	<0.001
SerpinC1	1.4 (1.2)	0.4 (0.8)	0.492	0.489
B. SERUM				
VWF	319 (667)	507 (488)	0.842	0.759
Plasminogen	67 (17)	23 (29)	0.024	0.018
SerpinC1	−36 (94)	−275 (55)	0.032	0.028

Absolute change in (A) LDL-EV coagulation protein and (B) serum coagulation proteins levels after two year placebo or rosuvastatin therapy. Changes in protein levels of VWF and plasminogen (in $\mu\text{g/mL}$ serum) and SerpinC1 (in mg/mL serum) are shown as mean (Standard Error of the mean).

Abbreviations: LDL: Low-density lipoprotein; EV: Extracellular vesicle; VWF: von Willebrand factor

* p-value results from the comparison of change in protein levels between placebo and rosuvastatin group, derived from univariable and multivariable linear regression analysis for all proteins except for serum plasminogen levels. Changes in serum plasminogen levels were analyzed using quantile regression analysis, because assumptions of linear regression were not met. Multivariable analyses are corrected for age, gender, body mass index, smoking status and hypertension. Values in bold are statistically significant ($p < 0.05$).

Plasminogen levels in serum also increased after 2 years of rosuvastatin treatment, but significantly less compared to placebo patients. This highlights that rosuvastatin has a different effect on plasminogen in LDL-EVs compared to plasminogen in serum, and any anticoagulant effect of rosuvastatin may be better reflected by the EV rather than serum levels, although this is only a hypothesis which remains to be elaborated in future studies.

4.2. Von Willebrand factor

VWF is a procoagulant protein that carries factor VIII, promotes thrombus formation via platelet aggregation and is an indicator for endothelial dysfunction [26,27]. Some studies have indicated that higher levels of VWF are associated with CVD, while others found no significant associations [23,28,29].

In our study we found increased levels of procoagulant VWF in LDL-EVs after rosuvastatin treatment. The underlying mechanism of this observation remains unclear, but one possible explanation is that LDL-EVs may act as carriers of encrypted VWF. Statins have been shown to improve the function of endothelial cells [24]. It is possible that statin therapy stimulates endothelial cells to excrete VWF encapsulated in EVs in contrast to directly into serum. When encapsulated by EVs, VWF may remain latent. This is only a hypothesis, which needs to be explored in future studies. Nevertheless, higher levels of VWF encapsulated in LDL-EVs might be a sign of improved endothelial function in patients using statins.

Rosuvastatin treatment did not affect serum levels of VWF in our cohort, which is consistent with previously published studies [30,31].

4.3. SerpinC1

SerpinC1, also known as anti-thrombin, is an anticoagulant protein and a major inhibitor of thrombus formation [32,33]. Both high and low levels of SerpinC1 being associated with increased risk of ischemic

Table 3
Relation between LDL reduction and LDL-EV and serum protein levels.

	VWF		Plasminogen		SerpinC1	
	Beta	95% CI	Beta	95% CI	Beta	95% CI
A. LDL-EV						
Univariable	1.80	−0.07–3.67	1.32	1.04–1.59	0.001	−0.026–0.028
Multivariable ^a	1.64	−0.26–3.54	1.34	1.06–1.62	0.002	−0.026–0.029
Multivariable + rosuvastatin ^b	0.08	−2.62–2.79	1.24	0.84–1.64	0.017	−0.022–0.056
Rosuvastatin and placebo group separately						
Rosuvastatin: univariable	0.04	−3.20–3.29	1.17	0.73–1.61	0.019	−0.023–0.062
Rosuvastatin: multivariable ^a	−0.32	−3.62–2.98	1.20	0.76–1.65	0.021	−0.022–0.064
Placebo: univariable	1.55	−2.75–5.85	1.37	0.44–2.30	−0.05	−0.099–0.090
Placebo: multivariable ^a	1.60	−2.74–5.94	1.35	0.40–2.29	−0.05	−0.101–0.092
B. SERUM						
Univariable	−2.07	−18.78–14.64	−0.15	−0.46–0.22	−2.95	−4.91 to −0.99
Multivariable ^a	−1.54	−18.47–15.40	−0.10	−0.48–0.27	−3.17	−5.12 to −1.21
Multivariable + rosuvastatin ^b	−6.34	−29.63–16.95	0.37	−0.09–0.83	−3.13	−5.81 to −0.44
Rosuvastatin and placebo group separately						
Rosuvastatin: univariable	−7.48	−33.74–18.78	0.40	−0.17–0.80	−2.93	−5.88–0.02
Rosuvastatin: multivariable ^a	−8.74	−35.40–17.93	0.32	−0.20–0.85	−3.35	−6.30 to −0.40
Placebo: univariable	1.62	−47.12–50.37	0.27	−0.80–1.33	−1.98	−8.83–4.87
Placebo: multivariable ^a	5.63	−44.04–55.31	0.04	−0.80–1.62	−2.55	−9.40–4.29

Results of univariable and multivariable regression analysis for the relation between LDL reduction and changes in protein levels in (A) LDL-EVs and (B) serum. Beta coefficients and 95% confidence interval were derived from linear regression analysis for all proteins except for serum plasminogen levels, which were derived from quantile regression analysis, because assumptions of linear regression were not met. Beta coefficients of VWF and plasminogen represent change in µg/mL per 1 mg/dL LDL. Beta coefficients of SerpinC1 represent change in mg/mL per 1 mg/dL change in LDL. Values in bold are statistically significant ($p < 0.05$).

Abbreviations: LDL: Low-density lipoprotein; EV: Extracellular vesicle; VWF: von Willebrand factor; Beta: Beta coefficient; 95% CI: 95% confidence interval

^a Corrected for age, gender, body mass index, smoking status and hypertension.

^b Corrected for age, gender, body mass index, smoking status, hypertension and rosuvastatin treatment.

heart disease [34,35]. This may be explained by the fact that SerpinC1 activity varies with the severity of atherosclerosis [35].

In the current study we found no association between LDL-EV-SerpinC1 levels and rosuvastatin treatment or LDL reduction. On the contrary, rosuvastatin treatment and LDL reduction were associated with a decrease in anti-thrombin levels in serum. This observation is hard to interpret without information on thrombin levels, because decrease in anti-thrombin levels could be a reflection of a decrease in thrombin activity, as statins have been shown to reduce thrombin generation and activity [8,24].

In conclusion, more research into the precise extent and nature of the effect of statins on extracellular vesicles containing coagulation proteins is needed in order to understand the underlying mechanisms involved. It would for example be very informative to investigate the effects of changes in LDL-EV coagulation proteins after statin therapy on actual thrombotic status or (markers for) cardiovascular events in future studies. This study does show that the effect of rosuvastatin on coagulation proteins in LDL-EVs differs from the effect on the same proteins in serum. This emphasizes the possible intermediary role of extracellular vesicles in the anticoagulation profile of statins.

4.4. Density gradient experiment

To investigate whether the coagulation proteins used in this study were derived from the EV fraction or from the lipoprotein particles, we conducted a density gradient experiment (supplementary methods). This experiment showed that VWF, plasminogen and SerpinC1 were abundantly present in subfractions with densities above 1.0 (fractions V6–V8, supplementary Fig. S2). As shown previously, EVs are present in the same subfractions [36]. In contrast, the levels of VWF, plasminogen and SerpinC1 were low in the apolipoprotein B positive lipid fraction (fraction V4, supplementary Fig. S2). Given that apolipoprotein B is the marker for LDL [37], this density gradient experiment, combined with electron microscopy results published previously [36], suggests that coagulation proteins were derived from the EV fraction and not from the lipoprotein particles. This is in line with the results of a recent publication of Wang et al. [36].

4.5. Strengths and limitations

Strengths of this study include the randomized controlled study design and the large sample size. This study also has several limitations. First, the design of the METEOR study did not include isolation of extracellular vesicles and therefore it cannot be ruled out that sample handling affected vesicle numbers and content. However, experiments have shown that freezing and thawing samples before vesicle isolation does not influence EV protein concentrations [38] and the possible misclassification would apply for both the rosuvastatin and placebo groups. Second, extracellular vesicles are preferably isolated from plasma instead of serum, since the coagulation process to obtain serum might cause vesicle release. Since plasma samples were not available, serum samples were used instead. It should be noticed that our analyses pertained to the difference in serum protein levels between two time points. The effect of using serum samples instead of plasma samples hence applies to both time points, and by using the difference between those time points we minimize the bias introduced by using serum instead of plasma. Using serum samples also restricted the present study to changes in coagulation proteins. If plasma samples had been available, the effect of coagulation protein changes on thrombotic status could have been studied as well. The present study was restricted to LDL-EVs and to three coagulation proteins, which only cover a small part of the total coagulation process and pathway. Although we cannot draw conclusions on the impact of the LDL-EV protein level changes on the overall thrombotic status, this study does show that statin treatment affects LDL-EVs and its coagulation protein content.

5. Conclusion

Rosuvastatin treatment significantly increased the LDL-EV levels of coagulation proteins VWF and plasminogen in patients with subclinical atherosclerosis, while this effect was different for serum values of plasminogen and absent for serum VWF. This identifies LDL-EVs as a newly detected possible intermediate between statin therapy and coagulation.

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Disclosures

Dr. Raichlen reported being an employee of AstraZeneca. Dr. Grobbee reported receiving grant support from and delivering lectures for Pfizer, AstraZeneca, Organon, Servier, and Merck. Dr. Bots reported receiving study grants for studies on carotid intima-media thickness and/or honoraria for professional input on carotid intima-media thickness issues from AstraZeneca, Icelandic Heart Foundation, Organon, Pfizer, the Netherlands Heart Foundation, the Netherlands Organization for Health Research and Development, Servier, and Unilever.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.ijcard.2018.05.098>.

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