International Journal of Cardiology xxx (2017) xxx–xxx

Contents lists available at ScienceDirect

International Journal of Cardiology

journal homepage: www.elsevier.com/locate/ijcard

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

Laura Verbree-Willemsen [a,1,2], Ya-Nan Zhang [b,1,2], Crystel M. Gijsberts [c,d,2], Arjan H. Schoneveld [c,2], Jiong-Wei Wang [b,2], Carolyn S.P. Lam [e,2], Floor Vernooij [b,2], Michiel L. Bots [a,2], Linda M. Peelen [a,f,2], Diederick E. Grobbee [a,2], Joel S. Raichlen [g,2], Dominique P.V. de Kleijn [b,c,d,h,*,2], On behalf of the METEOR Study Group

[a] Julius Center for Health Sciences and Primary Care, University Medical Center Utrecht, Utrecht University, The Netherlands
[b] Department of Surgery, Yong Loo Lin School of Medicine, National University of Singapore, Singapore & Cardiovascular Research Institute, National University Heart Centre Singapore, Singapore
[c] Experimental Cardiology Laboratory, University Medical Center Utrecht, Utrecht University, The Netherlands
[d] Interuniversity Cardiology Institute of the Netherlands, Utrecht University, The Netherlands
[e] National Heart Centre Singapore, Duke-NUS Graduate Medical School, Singapore & Cardiology, University Medical Center Groningen, Groningen, The Netherlands
[f] Department of Anesthesiology, University Medical Center Utrecht, Utrecht University, the Netherlands
[g] Clinical Department, AstraZeneca, Wilmington, DE, USA
[h] Department of Vascular Surgery, University Medical Center Utrecht, Utrecht University, The Netherlands

ARTICLE INFO

Article history:
Received 1 March 2018
Received in revised form 26 April 2018
Accepted 23 May 2018
Available online xxxx

Keywords:
Coagulation
Statin
Extracellular vesicles
Low-density lipoprotein
Plasminogen
von Willebrand factor

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 μg/mL for plasminogen ($p < 0.001$) and 310 ± 60 versus 64 ± 55 μg/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 μg/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.

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.

* Corresponding author at: Dept. of Vascular Surgery G04 129, Heidelberglaan 100, 3584 CX Utrecht, The Netherlands.
E-mail address: dkleijn@umcutrecht.nl (D.P.V. de Kleijn).
[1] These authors contributed equally to this work.
[2] This author takes responsibility for all aspects of the reliability and freedom from bias of the data presented and their discussed interpretation.

https://doi.org/10.1016/j.ijcard.2018.05.098
0167-5273/

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 ± 12 μg/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 ± 55 μg/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

Please cite this article as: L. Verbree-Willemsen, et al., LDL extracellular vesicle coagulation protein levels change after initiation of statin therapy. Findings from the METEOR trial, Int J Cardiol (2017), https://doi.org/10.1016/j.ijcard.2018.05.098