

Research Article

No Evidence for Statin-induced Proteinuria in Healthy Volunteers as Assessed by Proteomic Analysis

Anja Verhulst, Hilde Geryl, and Patrick D'Haese

Laboratory of Pathophysiology, University of Antwerp, Universiteitsplein 1, 2610 Antwerpen, Belgium

Correspondence should be addressed to Patrick D'Haese, patrick.dhaese@ua.ac.be

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In clinical studies of statins (class of drugs lowering plasma cholesterol levels), transient low-molecular-weight proteinuria was observed. The causes of statin-induced proteinuria in the patient background of those studies (cardiovascular and kidney disease) are multifactorial and, therefore, a matter of debate. In light of this, it seemed interesting to investigate the effect of statins on the urinary protein concentration and proteome in healthy volunteers. Six healthy volunteers were randomly treated with rosuvastatin (40 mg/day) or pravastatin (80 mg/day) in a double-blinded cross-over study. Total urinary protein concentration and the concentration of albumin/retinol-binding protein were analysed, after which the urinary proteome was investigated. From the results described in this study, it was concluded that statins do not induce major changes in the urinary protein concentration/proteome. High variability in the baseline urinary proteome/proteins among volunteers, however, made it very difficult to find subtle (possibly isolated to individuals) effects of statins.

1. Introduction

Statins, by their ability to inhibit 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, the rate-limiting enzyme of the sterol pathway, are potent inhibitors of sterol biosynthesis [1]. As a result of the reduction of cellular sterol pools, there is compensatory upregulation of cell-surface receptors for cholesterol-containing-low density lipoproteins (LDL), an effect that takes place mainly in the liver [2–4]. This mechanism underlies the therapeutic use of the statins to lower plasma cholesterol and particularly the levels of LDL. However, many additional effects of statins on cell function have been described in the literature [5]. These appear to be independent of cellular cholesterol homeostasis and are collectively termed “pleiotropic effects”. Many of these have been shown to result from the depletion of mevalonate- (the HMG-CoA conversion product) derived intermediates of the sterol pathway, particularly the isoprenoid pyrophosphates such as geranylgeranyl pyrophosphate (GGPP). Isoprenoid pyrophosphates are required by the cells for the posttranslational modification of a range of proteins, especially GTP-binding proteins.

In phase III studies of rosuvastatin, which included comparative studies with other statins and placebo, proteinuria

was observed in some subjects, most frequently in those taking rosuvastatin at the 80 mg dose (above the approved dose range of 5 to 40 mg). The proteinuria observed with rosuvastatin was generally transient, not associated with worsening renal function, and mainly of tubular type, suggesting reduced reabsorption of normally filtered proteins of low molecular weight [6–8]. This was further supported by results obtained in (human and opossum) renal epithelial cell cultures, in which receptor-mediated endocytosis could be inhibited by statins. Moreover, this effect could be prevented by the addition of mevalonate and GGPP but not cholesterol [9, 10]. The mechanism underlying this reduced rate of protein reabsorption was linked to inhibition of HMG-CoA reductase in the proximal tubule cells which in turn leads to a depletion of the cellular GGPP pool and thereby to reduced function of one or more GTP-binding proteins, known to be involved in the process of endocytosis [10–13].

To further explore the clinical relevance of these findings, the possible effect of statin treatment on the urinary protein composition of healthy volunteers randomly treated with the currently permitted doses of rosuvastatin (40 mg/day) or pravastatin (80 mg/day) was studied in a blinded cross-over

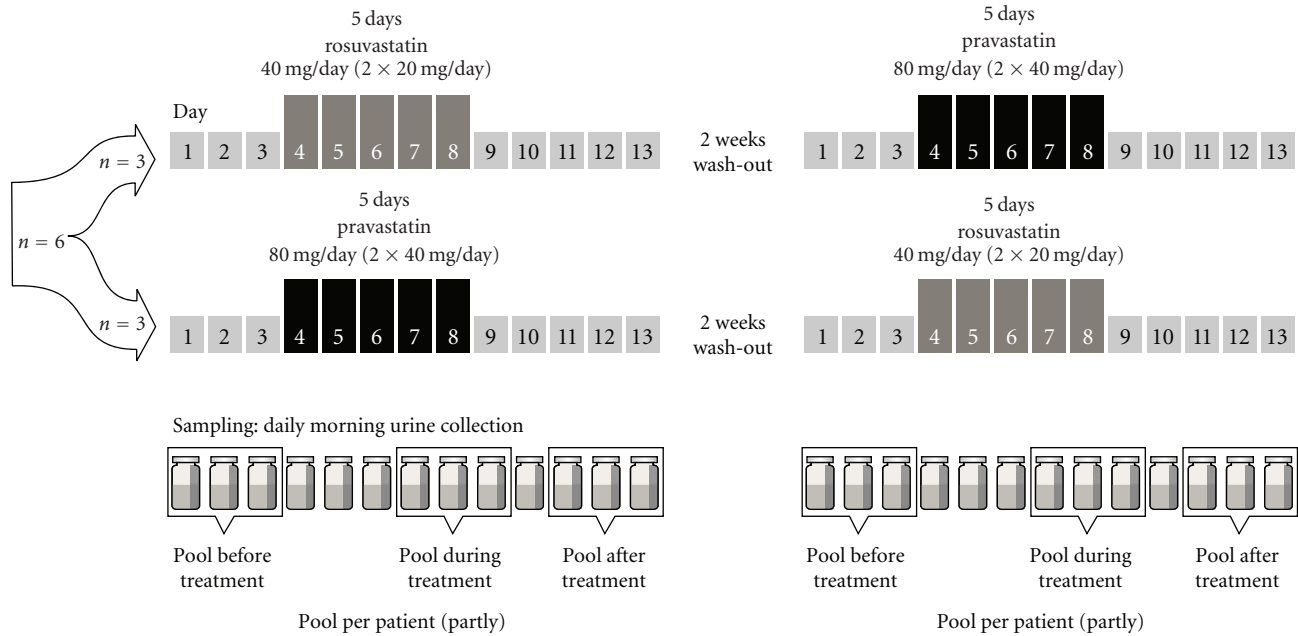


FIGURE 1: Schematic overview of the study setup.

study. Both pravastatin and rosuvastatin have a higher degree of renal secretion than the other marketed statins [14]. In the first instance, the total urine protein concentration and the concentration of albumin and retinol-binding protein in urine were analysed as accepted indices of the effect of the statins on tubular reabsorption of urinary proteins. Subsequently, the urinary proteome was investigated by two-dimensional gel-electrophoresis-based proteomics in order to investigate possible statin-induced effects on proteinuria in more detail.

2. Material and Methods

2.1. Study Setup and Urine Sampling. A blinded comparator cross-over study was performed (see Figure 1). Mid-stream morning urine was collected from 6 healthy volunteers (inclusion/exclusion criteria see Table 1) during two consecutive periods of 13 days, during which volunteers were treated (for 5 days) with a statin (rosuvastatin 40 mg/day; pravastatin: 80 mg/day) between 9 and 11 pm. Volunteers were recruited prospectively and started the study at the same moment. For 2D DIGE (2-D Fluorescence Difference Gel Electrophoresis) analysis, a number of 4 biological replicates is recommended in general. Since we were aware of the relatively high biological variation of proteinuria (both inter- and intravolunteer), we opted to work with 2 extra replicates (6 instead of 4 volunteers). A 2-week wash-out period without urine sampling was included between the two treatment periods. Three volunteers first received rosuvastatin followed by treatment with pravastatin, while the other three volunteers first underwent pravastatin treatment followed by rosuvastatin. Statin treatment started at day 4 and ended at day 8 of each urine collection period. In

this way, the statin treatment period was preceded by a 3-day (days 1–3) pretreatment period and followed by a 5-day off statin treatment period (days 9–13).

Urine samples consisted of fasting morning mid-stream urine which was collected in a sterile recipient already containing a protease-inhibitor tablet (Complete, Roche). The samples were stored by the participants in cool boxes and transported to the lab within 3 h after collection. Upon arrival, the samples were aliquoted in 3 fractions and stored at -80°C : two 10 mL aliquots were used for biochemical analysis and the remaining volume for proteome analysis.

Urine samples for proteome analysis were first prepared for CyDye labeling (see Section 2.3.1), after which they were pooled per volunteer and per treatment period as follows: before-treatment sample (samples of days 1 to 3), during-treatment sample (samples of days 7 to 9), and after-treatment sample (samples of days 11 to 13). Sample pooling resulted in a total of 36 samples (6 samples per volunteer). Samples of day 4, 5, 6, and 10 were not used for further analysis.

Informed consent of all 6 volunteers was obtained. Furthermore, this study was approved by the ethical committee of the Antwerp University Hospital, carried out in accordance with the code of ethics of the world medical association for experiments involving humans (declaration of Helsinki) and registered to EUDRACT.

2.2. Biochemical Urine Analysis. Urinary creatinine was determined by a colorimetric method (Creatinine Merckotest, Diagnostica Merck) based on Jaffe's method. Urinary protein content was measured using the Bradford method. Urinary microalbuminuria was analysed by nephelometry, using N-antiserum to human albumin. Finally, urinary

TABLE 1: Inclusion and exclusion criteria of the study.

Inclusion criteria	Exclusion criteria
Male	Treatment with lipid-lowering drugs <1 year prior to the study
Age 25–65 years	Known history of diabetes or fasting glucose level >110 mg/dL
Nonsmoker	Antihypertensive medication
Proteinuria <150 mg/24 hours	Lif expectancy <1 year
Dipstick negative hematuria	Pharmacological treatment with inotropes
Blood pressure <135 mm systolic, <85 mm diastolic	Acute or chronic inflammatory process, use of anti-inflammatory drugs, or immunosuppression
Waist circumference <94 cm	Clinically active malignant disease
	Administration of any investigational drug within 30 days preceding the study start and during the study
	Known intolerance to rosuvastatin or other statins
	Acute or chronic liver disease or ALAT >2.0 × upper limit of normal (ULN) at enrolment visit.
	Chronic muscle disease such as dermatomyositis or polymyositis or unexplained creatinine kinase (CK) above 3 × ULN at enrolment
	Uncontrolled hypothyroidism as indicated by a thyroid-stimulating hormone (TSH) >2 × ULN at enrolment
	Renal insufficiency: creatinine >2.0 mg/dL
	Known or suspect alcohol or drug abuse

retinol-binding protein concentration was determined using an ELISA-based method at the Laboratory of Toxicology of the University Hospital St-Luc, Brussels, Belgium.

2.3. Urinary Proteome Analysis Using 2D Fluorescence Difference Gel Electrophoresis (DIGE) Technology

2.3.1. Sample Preparation for CyDye Labeling. One hundred and eight urine samples (18 samples of 6 volunteers, see Section 2.1) were prepared for CyDye labeling. Urine samples were thawed at 25°C and centrifuged at 4°C for 10 min at 3000 rpm. Protein concentration was determined in the supernatant using the method of Bradford. Subsequently, the supernatant (no more than the volume corresponding to 1 mg of protein) was dialysed using dialysis membranes with a cut-off value of 3500 Da and polyethylene glycol 35000. After overnight dialysis at 4°C, the membranes were put in MQ H₂O for 15 min. The protein concentration of the remaining solution in the dialysis membrane was determined using the 2D quant kit (GE Healthcare) after which the 2D clean-up kit (GE Healthcare) was used to prepare the sample (150 µg of protein) for CyDye labeling. The protein concentration was determined again using the 2D quant kit. Finally, the pH of the sample was determined. All samples had a pH of 8.2 which fell within the allowed range of 7.0 to 9.4.

2.3.2. Pooling of the Samples. The 108 prepared samples were pooled per 3 samples (per volunteer and per treatment period, see Section 2.1), resulting in 36 pooled samples. Sample pooling was performed so that each of the 3 samples was equally present in the pooled sample, and resulted in a

50 µg protein sample. In addition, a pool of the 36 pooled samples was prepared which served as internal standard.

2.3.3. CyDye Labeling. The 36 pooled samples were labeled with Cy3 or Cy5. The internal standard was labeled with Cy2. CyDye (Cy2, Cy3, and Cy5, GE Healthcare) labeling was performed following manufacturer's instructions.

2.3.4. First Dimension (Isoelectric Focusing). The 36 Cy3- or Cy5-labeled samples were loaded onto 18 isoelectric focusing strips (Immobiline DryStrip with nonlinear pH gradient 3–10, GE Healthcare). Hereto each Cy3-labeled sample (50 µg) was at random added to a Cy5-labeled sample (50 µg) and to 50 µg of the Cy2 labeled internal standard. Subsequently, a mixture of 500 µL rehydration buffer (7 M urea, 2 M thio-urea, 1.5% CHAPS, 260 mM DTT and pharmalytes) and the Cy3-, Cy5-, and Cy2-labeled proteins was equilibrated for 6 hours on the isoelectric focusing strips. Isoelectric focusing was performed subsequently overnight, after which the strips were frozen at –80°C.

2.3.5. Second Dimension. Frozen strips were thawed and equilibrated for 20 min in 50 mM Tris-HCl buffer, pH 6.8, ureum 6 M, SDS 2%, and DTT 1%, followed by a further 20 min. equilibration in the same buffer with the exception that DTT 1% was replaced by iodoacetamide 4%. For the second dimension, 12.5% polyacrylamide gels were used.

2.3.6. Gel Scanning and Analysis. Gels were scanned on the Typhoon scanner (GE Healthcare) at 3 different excitation wavelengths: 488, 532 and 633 appropriate for Cy2, Cy3, and Cy5, respectively.

Analysis of the gels (each gel contains 3 spot maps, a Cy2-, a Cy3- and a Cy5-labeled), including the statistical analysis, was performed using ImageQuant and Decyder software (GE Healthcare). Spot protein identification was not a part of this study since statistical analysis of spot maps was not able to identify any protein with statistically significant expression in the different treatment groups.

3. Results

3.1. Biochemical Urine Analysis. The urinary protein content, microalbuminuria, and retinol-binding protein concentration were calculated per mg creatinine. A substantial variation was noticed for all three parameters, both with time in the same volunteer as among volunteers. However, all measurements of the three parameters fell within the normal range of these parameters. Also, no significant effect of statin treatment on any of these parameters could be observed (Figure 2).

3.2. Proteome Analysis

3.2.1. Assessment of Outlying Spot maps. In order to detect outlying spot maps that should be excluded for further analysis, principal component analysis of the 36 spot maps (6 spot maps of 6 volunteers) was performed. Since no outlying spot maps could be detected (see Figure 3), all 36 spot maps were included for further analysis.

3.2.2. Spot Detection. An average of 1852 ± 244 spots were detected on the spot maps. A master gel containing 1752 spots was chosen. Statistical analysis was performed on 965 spots (spots present on at least 70% of the spot maps).

3.2.3. General Effect of Statins on the Urinary Proteome. To check for a general effect of the statins under study on the urinary proteome, both principal component analysis and differential expression analysis were performed. Principal component analysis did not reveal any effect. Indeed, as demonstrated in Figure 4, no clustering of spot maps from either the “during-treatment period” or “before-” or “after-treatment” periods was observed. Differential expression analysis (one-way ANOVA and Student’s *t*-test, based on both paired and nonpaired statistics), with correction for multiple comparisons, also was not able to identify proteins that were differentially expressed as a result of statin treatment.

3.2.4. Inter- Versus Intravolunteer Variance. Principal component analysis of the spot maps of different volunteers made clear that spot maps of the same volunteers to a certain extent clustered (Figure 5). This could further be evidenced using hierarchical clustering analysis and differential expression analysis. Indeed, one-way ANOVA analysis, with correction for multiple comparisons, detected 276 (out of 965) proteins to be differentially expressed between volunteers with a *P* value <0.01 and 43 proteins (out of 965) to be differentially expressed between volunteers with a *P* value <0.001.

The fact that there was some clustering of spot maps in the individual volunteers indicates that the spot maps of a particular individual volunteer have more in common with each other than with the spot maps of other volunteers. In other words, the intervolunteer variance was higher compared with the intravolunteer variance. This prompted us to investigate a possible statin-induced effect in the different volunteers separately.

3.2.5. Intravolunteer Effect of Statins. Figure 5 clearly shows partial clustering of spot maps originating from one and the same volunteer. It was remarkable that nonclustering spot maps in most cases originated from the “during-treatment” period. In fact this was the case in 4 of the 6 volunteers. Figure 6 shows examples of nonclustering “during-treatment” spot maps. This intravolunteer approach, however, does not allow statistical analysis. Therefore, the analysis investigating the general effect in all volunteers was repeated, excluding the volunteers and treatment periods for which the “during-treatment” spot maps clustered (overlapped) with the “before-” and “after-” treatment spot maps. Principal component analysis showed partial clustering of the “during-treatment” spot maps (Figure 7). Differential expression analysis of these spot maps, however, did not identify statin-induced statistically significant differences in protein expression. A limited list of proteins with borderline significantly different expression during the treatment period lost significance when a correction for multiple comparison (false discovery rate correction) was made.

3.2.6. Effects of Different Treatment Arms. In order to separate the possible effects of the two different statins from each other we performed principal component analysis of the “during-treatment” spot maps of the two different statins under study (Figure 8). No statin-dependent clustering of spot maps could be observed.

This analysis was repeated, excluding the volunteers and treatment periods for which the “during-treatment” spot maps clustered with the “before-” and “after-” treatment spot maps. Again, this resulted in the absence of any statistically significant difference between “during-,” “before-,” and “after-treatment” spot maps for the two statins under study after correction for multiple comparison.

4. Discussion

The reported effects of statins on the kidney and the urinary protein concentration is multifactorial and, therefore, a matter of debate. Out of these discussions, one may conclude that the effect of statins on proteinuria is dual. (1) Patients treated with statins often suffer from underlying chronic kidney disease. Post hoc analysis of major statin trials (first endpoints were related to cardiovascular diseases) as well as meta-analyses of small randomized controlled trials suggest that statins reduce proteinuria and the rate of decline of glomerular filtration rate (for review see [15]). It was necessary to perform the current study in healthy volunteers which are free of this phenomenon, since it would complicate

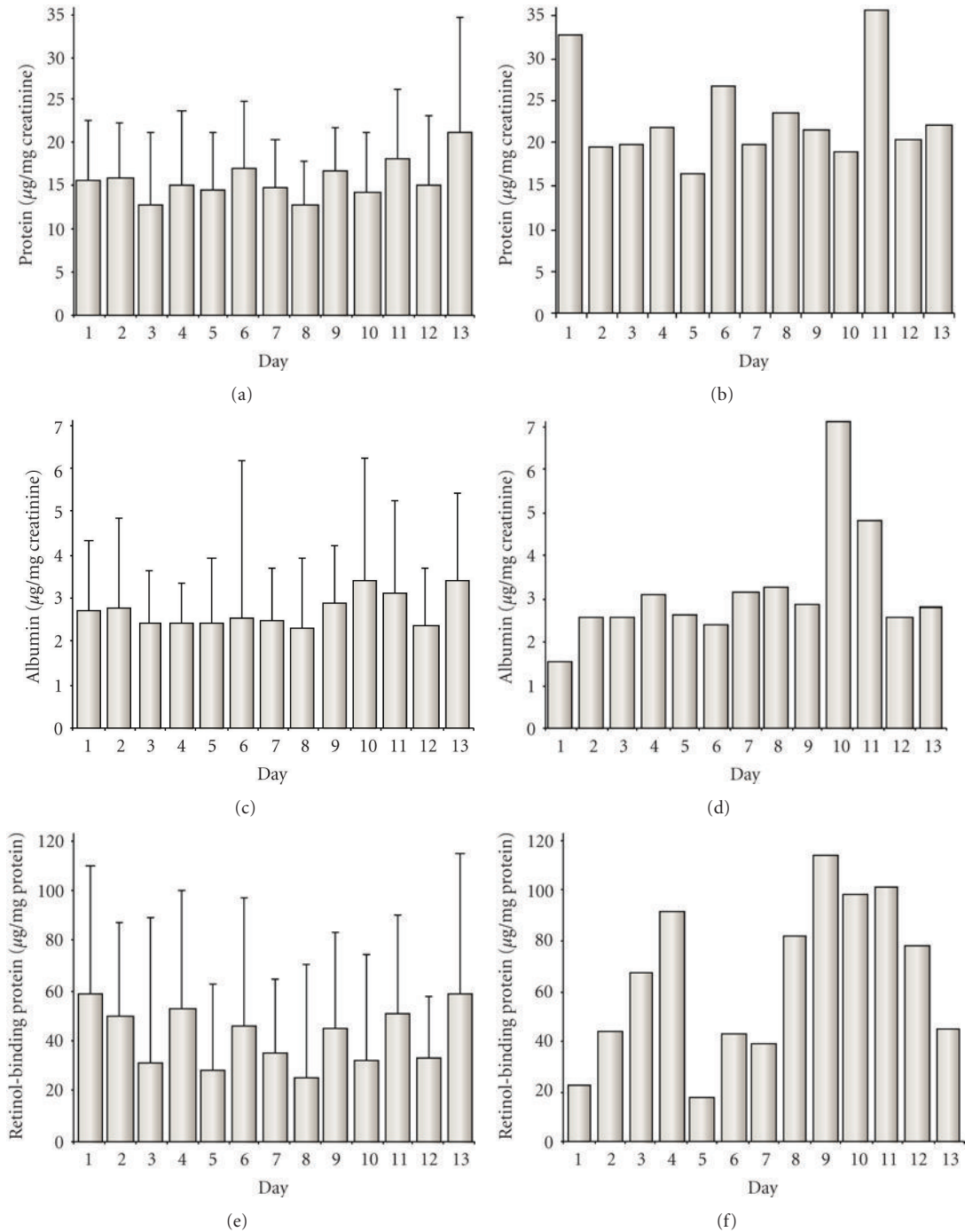


FIGURE 2: Quantification of proteins (a, b), microalbuminuria (c, d), and retinol binding protein (e, f) in urine. (a, c, e): mean values (and SD) of the urinary protein content of different volunteers and different treatment periods. (b, d, f): Urinary protein content of one representative volunteer during one-treatment period.

the investigation of a second phenomenon, completely unrelated to this but which we wanted to investigate. (2) This second phenomenon consists of the observation that statins, particularly at high doses, may increase proteinuria. As already described in the Introduction, this was observed during the preapproval studies of rosuvastatin: patients with the high 80 mg dose exhibited a higher incidence of

proteinuria on dipstick analysis compared with controls not receiving the compound. However, this increase in proteinuria was transient, did not lead to acute kidney injury or aggravate kidney failure [16], and was not associated with later development of renal impairment or renal failure [17].

The mechanism underlying this transient proteinuria was investigated *in vitro* in our laboratory and found to

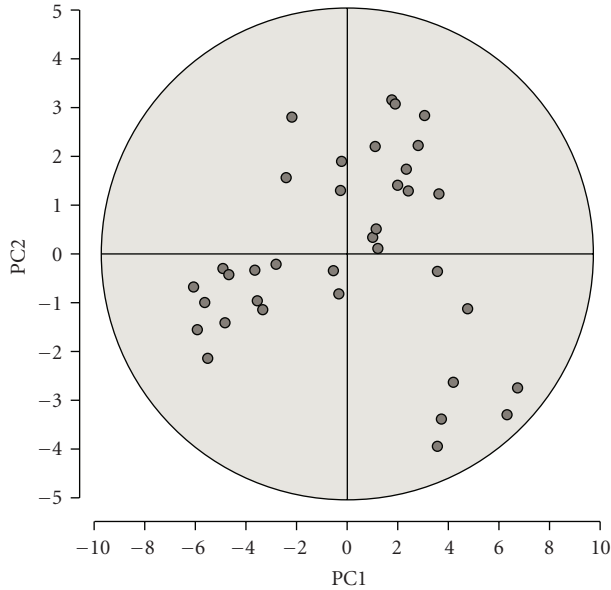
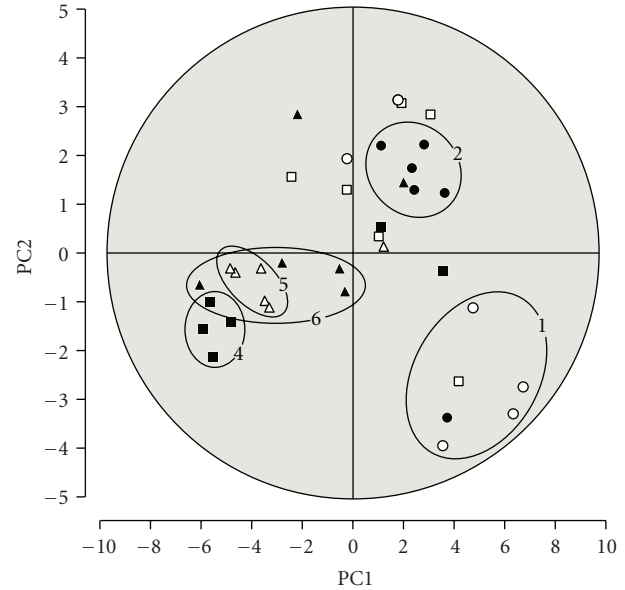


FIGURE 3: Principal component analysis of the 36 spot maps, representing the 36 samples originating of 6 volunteers. No outlying spot maps could be identified.



○ Volunteer 1
● Volunteer 2
□ Volunteer 3
■ Volunteer 4
△ Volunteer 5
▲ Volunteer 6

FIGURE 5: Principal component analysis of spot maps originating from different volunteers. Spot maps of the same volunteer to a certain extent cluster (ovals).

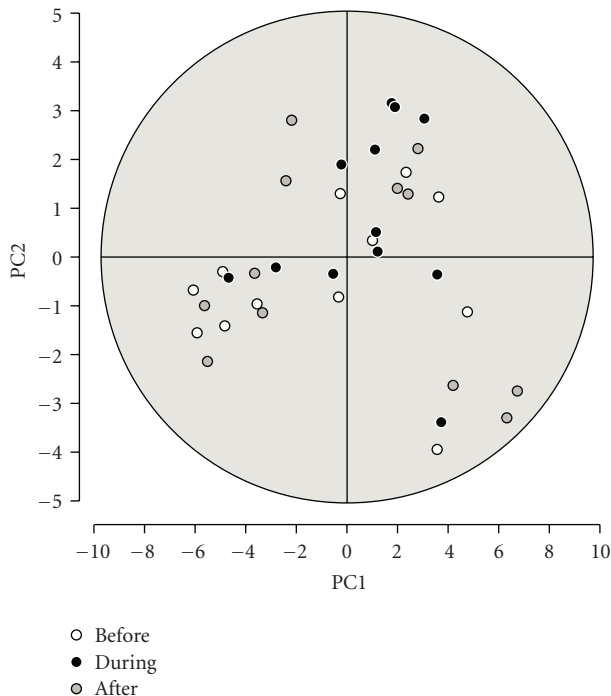


FIGURE 4: Principal component analysis of “before” “during,” and “after-treatment” spot maps. No clustering of spot maps can be observed for any if the 3 conditions.

be related to reduced proximal tubular protein endocytosis because of proximal tubular mevalonate depletion and reduced protein geranylgeranylation [9].

In line with this, it was observed that patients with primary hyperlipidemia taking rosuvastatin 10 or 20 mg/day for 12 weeks showed a concentration-dependent increase in the urinary α -1 microglobulin (a urinary low-molecular-weight protein) excretion of 17.6 and 34.9%, respectively [18].

One may speculate about the clinical effect of statin-induced inhibition of proximal tubular protein endocytosis. Overload of tubular cells with filtered proteins due to increased glomerular permeability as seen in progressive nephropathies has been put forward as the most important mechanism that translates glomerular protein leakage into tubular signals of interstitial inflammation and fibrosis [19–22]. In light of this, it may be speculated that statin use may be associated with less tubulointerstitial inflammation and fibrosis. Indeed, an *in vitro* study of Chana et al. made clear that statins are able to attenuate albumin-mediated chemokine production by proximal tubular cells. To induce this reduction; however, it was insufficient to inhibit the endocytosis of albumin alone [23]. Nonetheless, these results are congruent with the known pleiotropic anti-inflammatory actions of statins that are independent of lipid-lowering effects but largely dependent upon inhibition of NF κ B [24, 25]. Overall, the final outcomes of anti-NF κ B actions of statins are similar to their inhibiting effect on endocytosis and are also related to mevalonate depletion and reduced protein geranylgeranylation [26, 27].

Altogether, these observations encouraged us to investigate the effect of statin treatment on the urinary proteome, next to the measurement of the total urinary protein

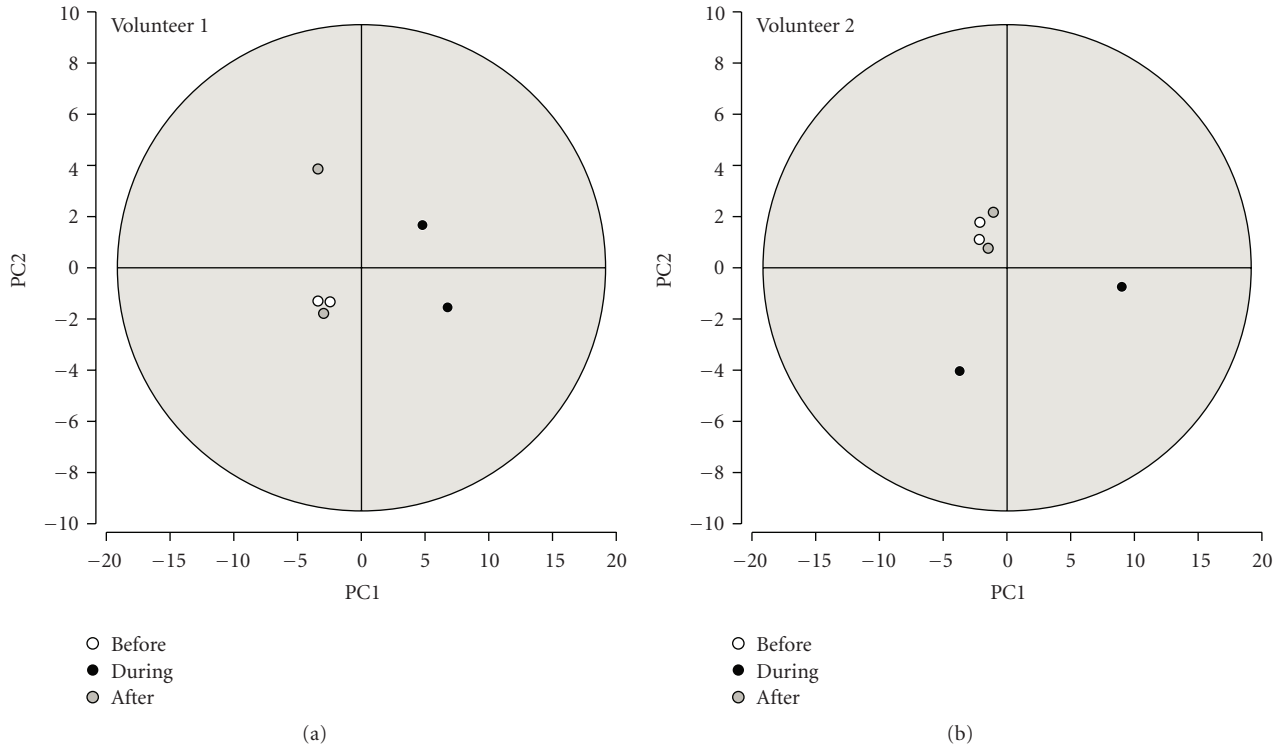


FIGURE 6: Principal component analysis of before-, during- and after-treatment spot maps of 2 different volunteers (representative examples).

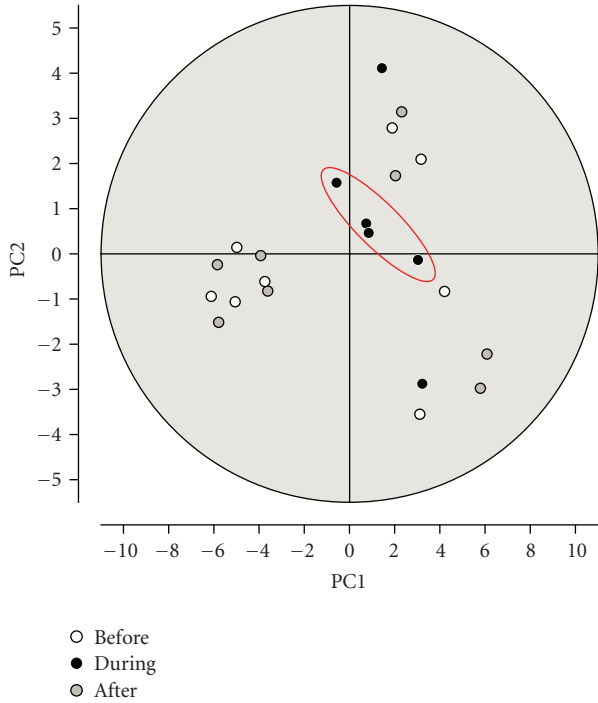


FIGURE 7: Principal component analysis of “before-”, “during-” and “after-treatment” spot maps. Partial clustering (red oval) of during-treatment spot maps can be observed.

and retinol-binding protein (low-molecular-weight protein) excretion in healthy volunteers, that is, without underlying kidney disease.

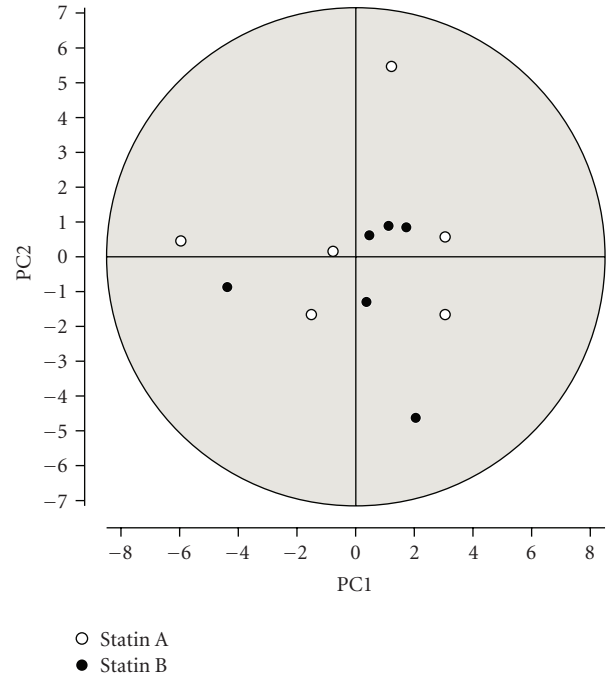


FIGURE 8: Principal component analysis of during-treatment spot maps originating from the two different statins: pravastatin (statin A) and rosuvastatin (statin B).

From the results described in this study, it could be concluded that statins do not induce important changes in the urinary protein concentration/proteome of healthy

volunteers. High variability in the baseline urinary proteome/proteins among volunteers, however, made it difficult to demonstrate distinct effects of statins. On the other hand, the fact that “during-treatment” spot maps of 4 of the 6 volunteers did not cluster with the “before-” and “after-” treatment spot maps when principal component analysis was performed, suggests that statins may exert subtle effects on the urinary proteome of some volunteers. Because of the limited number of subjects included in the current project (which was inherent to the currently used proteome analysis) and the above-described high variability in baseline proteome, this could not unequivocally be proven statistically.

Also, one cannot exclude the possibility that administration of a higher statin dose (rosuvastatin 80 mg/day instead of 40 mg/day) would have been necessary to induce a distinct effect on the protein concentration/proteome changes. At rosuvastatin doses ≤ 40 mg/day, the rate of dipstick positive proteinuria was within the range observed with other statins and, notably, placebo. However, administration of rosuvastatin at the 80 mg/day dose is not approved, and treating healthy volunteers at this dose is not ethically permissible. Because dipstick analysis (as used during the rosuvastatin phase III trials in which proteinuria was detected) is a highly insensitive measure for proteinuria; however, it was hoped that the greater sensitivity of proteomics would enable characterization of more subtle (subclinical) effects induced by statins at lower doses. In this context, it is also worth mentioning that few patients treated with rosuvastatin at 40 mg/day (2%) and a greater proportion of patients treated with 80 mg/day (33%) achieved a steady-state plasma drug concentration (<50 ng/mL), suggesting a potential threshold in the drug level at which the risk for distinct proteinuria is increased [14]. Although we expected to observe possible statin-induced effects already after a short treatment period, it is also possible that longer treatment periods are necessary to induce distinct effects. However, it was also not allowed for ethical reasons to treat healthy volunteers for longer periods with the high (highest allowed) statin doses.

In general, it can be concluded that short-term statin treatment with the highest allowed doses in healthy volunteers does not induce major changes in the urinary protein concentration/proteome.

Abbreviations

DIGE:	2D Fluorescence Difference Gel Electrophoresis
GGPP:	Geranylgeranyl pyrophosphate
HMG-CoA:	3-Hydroxy-3-methylglutaryl coenzyme A
LDL:	Low Density lipoprotein.

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