



## Proprotein convertase subtilisin–kexin type 9 is elevated in proteinuric subjects: Relationship with lipoprotein response to antiproteinuric treatment



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### ABSTRACT

**Objective:** LDL-receptor deficiency may provide a mechanism which contributes to atherogenic lipoprotein abnormalities in experimental nephrosis and in humans with glomerular proteinuria. The proprotein convertase subtilisin–kexin type 9 (PCSK9) pathway plays a key role in lipoprotein metabolism by promoting LDL-receptor degradation. We tested whether plasma PCSK9 is elevated in proteinuric states, and determined relationships of PCSK9 with lipoprotein responses to proteinuria reduction.

**Methods:** Thirty-nine kidney patients (e-GFR  $61 \pm 29$  mL/min/1.73 m<sup>2</sup>, proteinuria  $1.9 [0.9–3.3]$  g/day; 19 on statin treatment) were studied during 2 randomized double-blind 6-week periods on either lisinopril (40 mg/day) and a regular sodium diet ( $194 \pm 49$  mmol Na<sup>+</sup>/day; baseline treatment) or lisinopril plus valsartan (320 mg/day) and a low sodium diet ( $102 \pm 52$  mmol Na<sup>+</sup>/day; maximal treatment), and compared to age- and sex-matched controls. Maximal treatment decreased proteinuria to  $0.5 [0.3–1.1]$  g/day ( $P < 0.001$ ).

**Results:** Plasma PCSK9 was increased at baseline in proteinuric subjects ( $213 [161–314]$  vs.  $143 [113–190]$  ug/L in controls,  $P \leq 0.001$ ), irrespective of statin use, e-GFR and BMI. PCSK9 correlated with proteinuria at baseline ( $R = 0.399$ ,  $P = 0.018$ ) and at maximal antiproteinuric treatment ( $R = 0.525$ ,  $P = 0.001$ ), but did not decrease during proteinuria reduction ( $P = 0.84$ ). Individual changes in total cholesterol ( $R = 0.365$ ,  $P = 0.024$ ), non-HDL cholesterol ( $R = 0.333$ ,  $P = 0.041$ ), and LDL cholesterol ( $R = 0.346$ ,  $P = 0.033$ ) were correlated positively with individual PCSK9 responses. PCSK9 at baseline independently predicted the total/HDL cholesterol ratio response to treatment ( $P = 0.04$ ).

**Conclusion:** Plasma PCSK9 was elevated in proteinuria, predicted lipoprotein responses to proteinuria reduction but remained unchanged after proteinuria reduction. Inhibition of the PCSK9 pathway may provide a novel treatment strategy in proteinuric subjects.

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### 1. Introduction

Dyslipidemia is regarded an inherent feature of overt glomerular proteinuria [1–3]. Accordingly, higher total cholesterol, low density lipoprotein (LDL) cholesterol, very low density lipoprotein (VLDL) cholesterol (collectively designated as non-HDL cholesterol) and triglycerides are common in proteinuric subjects [1–3], whereas decreased high density lipoprotein (HDL) cholesterol levels have been reported as well [2–4]. Hence, the total

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cholesterol/HDL cholesterol ratio, which is considered a main lipid risk factor in the general population [5], is elevated in proteinuric conditions [6]. Moreover, it is likely that such elevations in apolipoprotein B (apoB)-containing lipoproteins are pathogenetically involved in the increased cardiovascular risk encountered in proteinuric subjects [6,7]. In this regard it is relevant that anti-proteinuric therapy improves at least in part the increases in apoB-containing lipoproteins attributable to proteinuria, even irrespective of treatment modality [2,3,6].

Despite intensive research, the complex mechanisms underlying the changes in lipoprotein metabolism in proteinuric states remain incompletely understood. Hypercholesterolemia consequent to hepatic LDL-receptor deficiency has been documented in experimental nephrosis [8]. In humans with non-diabetic nephrotic-range proteinuria, defective LDL apoB catabolism has been proposed to represent the primary metabolic abnormality [9,10]. However, using stable isotope techniques impaired VLDL apoB catabolism in combination with increased LDL apoB synthesis has been identified as the main metabolic abnormality in another study [11].

In the past few years it has increasingly been recognized that the proprotein convertase subtilisin–kexin type 9 (PCSK9) pathway plays a pivotal role in LDL-metabolism by modulating hepatic LDL-receptor expression [12,13]. PCSK9 is a secreted protease which binds to the extracellular domain of the LDL-receptor, where it acts as a chaperone that is able to target the LDL-receptor towards intracellular degradation, thereby preventing LDL-receptor recycling to the cell surface [13]. Indeed, a lower LDL apoB fractional catabolic rate is predicted by higher plasma PCSK9 levels in humans, suggesting that between-subject differences in circulating PCSK9 levels are clinically important [14]. Accordingly, apoB-containing lipoproteins levels are associated positively with the PCSK9 concentration [14–17]. Furthermore, gain-of-function mutations in PCSK9 are associated with hypercholesterolemia, whereas loss-of-function mutations relate to lower apoB levels and cardioprotection [13]. In view of diminished hepatic LDL-receptor expression in experimental nephrosis [8], and impaired LDL catabolism in humans with nephrotic range proteinuria [9,10], it is important to test whether the plasma PCSK9 level, as a measure of activation of the PCSK9 pathway, is increased in proteinuric states.

This study was initiated to determine whether plasma PCSK9 levels are elevated in humans with glomerular proteinuria compared to healthy subjects. We furthermore assessed relationships between lipoprotein responses during maximal anti-proteinuric treatment and PCSK9 levels in proteinuric subjects.

## 2. Material and methods

### 2.1. Study subjects

The study population consisted of 39 proteinuric subjects and 39 healthy controls.

Inclusion criteria for proteinuric subjects were proteinuria  $>1$  g/day during high dose angiotensin converting enzyme inhibition (ACEi), blood pressure  $>125/75$  mmHg, creatinine clearance  $\geq 30$  mL/min, and age  $\geq 18$  years. All proteinuric subjects were of Caucasian race. Exclusion criteria were systolic blood pressure  $\geq 180$  mmHg, diastolic blood pressure  $\geq 110$  mmHg, diabetes mellitus (using World Health Organization criteria [18]), renovascular hypertension, decrease of creatinine clearance  $\geq 6$  mL/min in the preceding year, cardiovascular event in the previous 6 months, immunosuppressive treatment, regular use ( $>1$  day/week) of non-steroidal anti-inflammatory drugs, and pregnancy. Renal diagnosis was focal segmental glomerulosclerosis ( $n = 13$ ), immunoglobulin A nephropathy ( $n = 13$ ), membranous nephropathy ( $n = 7$ ), hypertensive nephropathy ( $n = 2$ ) and other/inconclusive ( $n = 4$ ).

Caucasian healthy subjects (aged  $\geq 18$  years) were recruited by advertisement and served as controls. Diabetes mellitus, hypertension, proteinuria, renal function or thyroid impairment, and pregnancy were exclusion criteria. None of these subjects used any medication except for oral contraceptives. Healthy subjects were individually matched with proteinuric subjects with respect to age (within 5 years) and sex.

### 2.2. Study protocol

The current study was a secondary analysis among participants from a previously published study [19]. This study was a prospective randomized double-blind, placebo-controlled cross-over trial in which the effects of angiotensin receptor blockade (ARB) and dietary sodium restriction on proteinuria and blood pressure were evaluated in subjects with stable proteinuric states.

All proteinuric subjects were enrolled in a run-in period of at least 6-weeks in which subjects received background treatment with an ACEi at maximum dose (lisinopril 40 mg/day) while stopping other renin-angiotensin-aldosterone-system blockers. Subjects were subsequently treated with combinations of placebo, ARB (valsartan 320 mg/day), regular sodium diet (mean intake  $194 \pm 49$  mmol Na<sup>+</sup>/day) and a low sodium diet (mean intake  $102 \pm 52$  mmol Na<sup>+</sup>/day) during four random 6-week study periods. Additional antihypertensive medication was allowed but kept stable during the study. To test the hypothesis of the present study we used ACEi combined with regular sodium diet as the baseline treatment period, and ACEi combined with ARB and low sodium diet, which was documented to result in maximal antiproteinuric treatment [19], as the intervention period. Plasma samples for measurement of PCSK9 and (apo)lipoproteins from both study periods were available in 39 out of the original 52 study subjects. Age, sex, proteinuria, e-GFR, blood pressure and BMI were not different between the 39 subjects included in the present report compared to the 13 subjects not currently studied (data not shown).

The study protocol was approved by the local ethical committee of the University Medical Center Groningen, The Netherlands, and conducted according to guideline for good clinical practice and declaration of Helsinki. Written informed consent was obtained from each subject.

### 2.3. Measurements and calculations

Proteinuric subjects visited the outpatient nephrology clinic at end of each 6-week treatment period for clinical assessment. Blood pressure was measured for 15 min at 1-min intervals by an automatic device (Dinamap, G.E. Medical Systems, Milwaukee, WI, USA), with subjects in a supine position. We used the mean of the last three readings. Body mass index (BMI) was calculated by dividing body weight by height squared ( $\text{kg}/\text{m}^2$ ). Subjects collected 24-h urine one day prior to their visit to the outpatient clinic. To correct for sampling errors urinary protein/creatinine excretion was calculated as the ratio of urinary protein excretion and creatinine excretion. Estimated glomerular filtration rate (e-GFR) was calculated using CKD-EPI formula [20]. Absolute treatment responses were calculated by subtracting baseline treatment values from values at maximal antiproteinuric treatment.

### 2.4. Laboratory analyses

Blood was obtained after an overnight fast, collected in EDTA-containing tubes (1.5 mg/mL) and placed immediately on ice. Plasma was obtained by centrifugation at 3000 G for 10 min at 4 °C. Plasma glucose was measured shortly after blood sampling (APEC

Inc., Danvers, MA, USA) and samples for other measurements were stored at  $-80^{\circ}\text{C}$  until analysis.

Plasma cholesterol and triglycerides were assayed by routine enzymatic methods (Roche/Hitachi cat. no. 11876023 and 11875540, respectively). HDL cholesterol was measured using a homogeneous enzymatic calorimetric test (Roche/Hitachi cat. no. 03030024). Non-HDL cholesterol was calculated as total cholesterol minus HDL cholesterol. LDL cholesterol was calculated with the Friedewald formula. ApoB was measured by immunoturbidimetry (Boehringer Mannheim, Almere, The Netherlands, cat. no. 726494). Plasma PCSK9 was measured by ELISA as described elsewhere [21]. The inter-assay coefficient of variance of PCSK9 measurement was  $<6\%$ .

Serum and urinary creatinine was measured by an automated multianalyzer and urinary protein by a turbidimetric assay using benzethonium chloride (both Modular, Roche Diagnostics, Mannheim, Germany).

### 2.5. Statistical analysis

Data are given as mean with standard deviation (SD) when normally distributed, and otherwise as median with interquartile range (IQR). Skewed variables were log-transformed to obtain normality for analysis. Comparison between healthy subjects and proteinuric subjects were tested using independent *T*-tests for continuous variables and chi-squared tests for dichotomized variables. In proteinuric subjects, paired *T*-tests were used to determine effects of maximal antiproteinuric treatment. Univariate relationships were assessed by Spearman's correlation analysis. Multivariate regression analysis was used for independent relationships between variables. To account for the effect of statin treatment we introduced dummy variables with the healthy subjects as a reference category in the multivariate analysis. We also used sex-specific tertiles of PCSK9 levels at baseline treatment and applied two-way ANOVA with polynomial contrast to test presence of a trend with respect to lipoprotein changes in response to antiproteinuric treatment. Data were analyzed using SPSS version 18.0 (SPSS Inc., Chicago, IL). Two-sided *P*-values  $\leq 0.05$  were considered statistically significant.

**Table 1**  
Clinical characteristics in healthy subjects ( $n = 39$ ) and proteinuric subjects ( $n = 39$ ).

|   | Healthy subjects<br>( $n = 39$ ) | Proteinuric subjects ( $n = 39$ ) |                                   |
|---|----------------------------------|-----------------------------------|-----------------------------------|
|   |                                  | Baseline treatment                | Maximal antiproteinuric treatment |
| Male sex, $n$ (%)                                 | 34 (87%)                         | 34 (87%)                          |                                   |
| Age (years)                                       | $52 \pm 11$                      | $50 \pm 13$                       |                                   |
| Additional antihypertensive treatment, $n$ (%)    | 0 (0%)                           | 16 (41%)*                         |                                   |
| Statin treatment, $n$ (%)                         | 0 (0%)                           | 19 (49%)*                         |                                   |
| BMI ( $\text{kg}/\text{m}^2$ )                    | $25.4 \pm 3.0$                   | $27.8 \pm 3.5^{**}$               | $27.5 \pm 3.4^{**\#}$             |
| Glucose (mmol/L)                                  | $5.5 \pm 0.6$                    | $5.4 \pm 0.4$                     | $5.3 \pm 0.6$                     |
| Systolic blood pressure (mmHg)                    | $129 \pm 15$                     | $132 \pm 19$                      | $120 \pm 19^{\#\#\#}$             |
| Diastolic blood pressure (mmHg)                   | $83 \pm 9$                       | $78 \pm 13^*$                     | $70 \pm 15^{***\#\#\#}$           |
| Serum creatinine ( $\mu\text{mol}/\text{L}$ )     | $81 \pm 9$                       | $138 \pm 61^{***}$                | $162 \pm 66^{***\#\#\#}$          |
| e-GFR ( $\text{mL}/\text{min}/1.73 \text{ m}^2$ ) | $93 \pm 11$                      | $61 \pm 29^{***}$                 | $48 \pm 20^{***\#\#\#}$           |
| Serum albumin (g/L)                               | N.A.                             | 39 [36–42]                        | 41 [37–44]###                     |
| Proteinuria (g/day)                               | 0 [0–0]                          | 1.9 [0.9–3.3]***                  | 0.5 [0.3–1.1]***/###              |
| Urinary protein/creatinine ratio (mg/mg)          | 0 [0–0]                          | 1.0 [0.5–2.0]***                  | 0.4 [0.2–0.9]**/###               |

\* $P \leq 0.05$  vs. healthy subjects. \*\* $P \leq 0.01$  vs. healthy subjects. \*\*\* $P \leq 0.001$  vs. healthy subjects. # $P \leq 0.05$  vs. proteinuric subjects at baseline treatment. ## $P \leq 0.01$  vs. proteinuric subjects at baseline treatment. ### $P \leq 0.001$  vs. proteinuric subjects at baseline treatment.

Data are shown as mean  $\pm$  SD or as median (IQR). N.A.: not available. BMI: body mass index; e-GFR: estimated glomerular filtration rate.

### 3. Results

Characteristics of the proteinuric subjects and their age- and sex-matched healthy controls are shown in Table 1. Proteinuric subjects had a lower e-GFR ( $P < 0.001$ ) and higher BMI ( $P = 0.002$ ) at baseline compared with healthy subjects. Glucose was similar in both groups. Sixteen out of 39 (41%) proteinuric subjects were treated with 2 (range: 1–4) antihypertensives in addition to standardized background ACEi. In all proteinuric subjects, blood pressure was well-controlled in comparison to healthy subjects. Nineteen out of 39 (49%) proteinuric subjects were on stable statin treatment [atorvastatin (10–80 mg/day),  $n = 7$ ; simvastatin (20–40 mg/day) with or without ezetimibe (10 mg/day),  $n = 9$ ; pravastatin (20–80 mg/day),  $n = 2$ ; rosuvastatin, (5 mg/day),  $n = 1$ ] throughout the study. The median duration of statin treatment was 4 [3–9] years.

At baseline treatment, the total cholesterol/HDL cholesterol ratio was higher in all proteinuric subjects combined compared to healthy subjects. In proteinuric subjects, plasma triglycerides were also higher coinciding lower HDL cholesterol. Their total cholesterol was lower, but LDL cholesterol and non-HDL cholesterol levels were not different from healthy subjects (Table 2A). In non-statin treated proteinuric subjects separately, baseline total cholesterol, non-HDL cholesterol and LDL cholesterol levels were not significantly different from matched healthy subjects, but the total cholesterol/HDL cholesterol ratio was higher and HDL cholesterol was lower. In statin-treated proteinuric subjects, total cholesterol, LDL cholesterol and HDL cholesterol were lower compared to matched healthy subjects (Table 2B). It should be emphasized that among statin-treated proteinuric subjects, total cholesterol ( $8.53 \pm 2.08$ ,  $P < 0.001$ ), LDL cholesterol ( $5.78 \pm 1.43$ ,  $P < 0.001$ ), non-HDL cholesterol ( $7.66 \pm 2.25$ ,  $P = 0.001$ ) and the total cholesterol/HDL cholesterol ratio ( $7.88 \pm 2.12$ ,  $P = 0.002$ ) were much higher before initiation of statin therapy compared to baseline treatment.

At baseline treatment, plasma PCSK9 levels were higher in the total group of proteinuric subjects ( $n = 39$ ; 213 [161–314]  $\mu\text{g}/\text{L}$ ) compared to healthy subjects ( $n = 39$ ; 143 [113–190]  $\mu\text{g}/\text{L}$ ,  $P < 0.001$ ; Fig. 1). PCSK9 levels at baseline treatment were also higher in proteinuric subjects receiving statin treatment ( $n = 19$ ; 244 [185–360]  $\mu\text{g}/\text{L}$ ), as well as in proteinuric subjects not receiving statin treatment ( $n = 20$ ; 193 [140–240]  $\mu\text{g}/\text{L}$ ) compared to age- and

**Table 2A**  
Plasma lipoproteins in healthy subjects ( $n = 39$ ) and proteinuric subjects ( $n = 39$ ).

|   | Healthy subjects<br>( $n = 39$ ) | Proteinuric subjects ( $n = 39$ ) |                                   |
|---|----------------------------------|-----------------------------------|-----------------------------------|
|   |                                  | Baseline treatment                | Maximal antiproteinuric treatment |
| Total cholesterol (mmol/L)              | $5.59 \pm 1.15$                  | $5.01 \pm 1.22^*$                 | $4.69 \pm 1.11^{***\#\#}$         |
| Non-HDL cholesterol (mmol/L)            | $4.15 \pm 1.29$                  | $3.98 \pm 1.21$                   | $3.75 \pm 1.02^{\#}$              |
| LDL cholesterol (mmol/L)                | $3.40 \pm 1.04$                  | $2.96 \pm 1.16$                   | $2.83 \pm 0.94^*$                 |
| HDL cholesterol (mmol/L)                | $1.44 \pm 0.44$                  | $1.03 \pm 0.29^{***}$             | $0.94 \pm 0.25^{***\#\#\#}$       |
| Triglycerides (mmol/L)                  | 1.34 [0.92–1.90]                 | 1.83 [1.28–3.05]*                 | 1.88 [1.34–2.67]*                 |
| Total cholesterol/HDL cholesterol ratio | $4.31 \pm 1.74$                  | $5.14 \pm 1.58^*$                 | $5.18 \pm 1.3^*$                  |
| ApoB (g/L)                              | $0.96 \pm 0.29$                  | $1.01 \pm 0.35$                   | $0.98 \pm 0.30$                   |

\* $P \leq 0.05$  vs. healthy subjects. \*\* $P \leq 0.01$  vs. healthy subjects. \*\*\* $P \leq 0.001$  vs. healthy subjects. # $P \leq 0.05$  vs. proteinuric subjects at baseline treatment. ## $P \leq 0.01$  vs. proteinuric subjects at baseline treatment. ### $P \leq 0.001$  vs. proteinuric subjects at baseline treatment. Data are shown as mean  $\pm$  SD or as median (IQR). HDL cholesterol; high density lipoprotein cholesterol; LDL cholesterol; low density lipoprotein cholesterol; ApoB: apolipoprotein B.

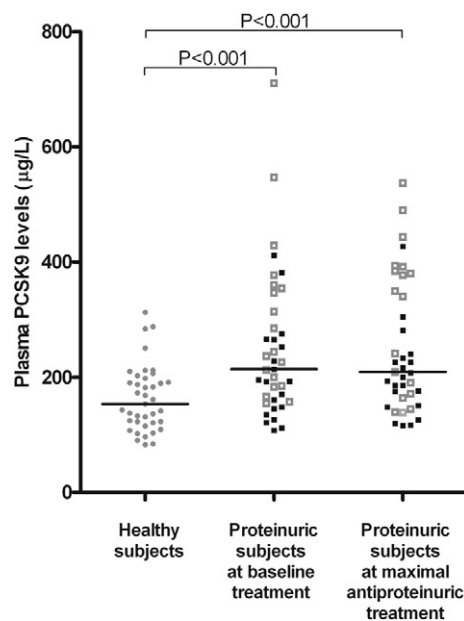
**Table 2B**Plasma lipoproteins in non-statin ( $n = 20$ ) and statin treated ( $n = 19$ ) proteinuric subjects and their individual matched healthy subjects.

|                                   | Healthy subjects matched with non-statin treated proteinuric subjects ( $n = 20$ ) | Proteinuric subjects not treated with statins ( $n = 20$ ) |                                   | Healthy subjects matched with statin treated proteinuric subjects ( $n = 19$ ) | Proteinuric subjects treated with statins ( $n = 19$ ) |                                   |
|-----------------------------------|--|--|-----------------------------------|--|--|-----------------------------------|
|                                   |  | Baseline treatment   | Maximal antiproteinuric treatment |  | Baseline treatment                                     | Maximal antiproteinuric treatment |
| Total cholesterol (mmol/L)        | 5.34 ± 1.17  | 5.33 ± 1.17  | 4.74 ± 0.83 <sup>###</sup>        | 5.85 ± 1.09  | 4.68 ± 1.21 <sup>**</sup>                              | 4.63 ± 1.38 <sup>**</sup>         |
| Non-HDL cholesterol (mmol/L)      | 3.94 ± 1.29  | 4.29 ± 1.22  | 3.84 ± 0.83 <sup>##</sup>         | 4.36 ± 1.28  | 3.65 ± 1.14  | 3.65 ± 1.22                       |
| LDL cholesterol (mmol/L)          | 3.24 ± 1.15  | 3.36 ± 1.10  | 3.02 ± 0.77 <sup>#</sup>          | 3.57 ± 0.93  | 2.54 ± 1.08 <sup>**</sup>                              | 2.62 ± 1.08 <sup>**</sup>         |
| HDL cholesterol (mmol/L)          | 1.40 ± 0.41  | 1.04 ± 0.30 <sup>***</sup>                                 | 0.90 ± 0.22 <sup>###</sup>        | 1.49 ± 0.48  | 1.03 ± 0.28 <sup>***</sup>                             | 0.98 ± 0.28 <sup>***</sup>        |
| Triglycerides (mmol/L)            | 1.49 [0.90–1.74]   | 1.62 [1.02–2.49]   | 1.69 [1.21–2.47]                  | 1.10 [0.92–2.07]   | 1.90 [1.29–3.16]                                       | 2.06 [1.51–3.02] <sup>*</sup>     |
| Total cholesterol/HDL cholesterol | 3.81 [2.72–5.57]   | 5.79 [3.92–6.83] <sup>*</sup>                              | 5.87 [4.29–6.28] <sup>**</sup>    | 3.93 [2.84–6.04]   | 4.89 [4.08–5.98]                                       | 4.80 [4.02–5.25]                  |
| ApoB (g/L)                        | 0.93 ± 0.30  | 1.10 ± 0.29  | 1.01 ± 0.22 <sup>#</sup>          | 0.99 ± 0.28  | 0.92 ± 0.38  | 0.94 ± 0.36                       |
| PCSK9 (µg/L)                      | 137 [109–188]  | 193 [140–240] <sup>*</sup>                                 | 197 [150–230] <sup>**</sup>       | 153 [116–198]  | 244 [185–360] <sup>***</sup>                           | 344 [172–392] <sup>***</sup>      |

<sup>\*</sup> $P \leq 0.05$  vs. healthy subjects. <sup>\*\*</sup> $P \leq 0.01$  vs. healthy subjects. <sup>\*\*\*</sup> $P \leq 0.001$  vs. healthy subjects. <sup>#</sup> $P \leq 0.05$  vs. proteinuric subjects at baseline treatment. <sup>##</sup> $P \leq 0.01$  vs. proteinuric subjects at baseline treatment. <sup>###</sup> $P \leq 0.001$  vs. proteinuric subjects at baseline treatment. Data are shown as mean ± SD or as median (IQR). HDL cholesterol; high density lipoprotein cholesterol; LDL cholesterol: low density lipoprotein cholesterol; ApoB: apolipoprotein B; PCSK9: proprotein convertase subtilisin–kexin type 9.

sex-matched healthy subjects separately ( $n = 19$ ; 153 [116–198],  $P < 0.001$ ; and  $n = 20$ ; 137 [109–188] µg/L,  $P < 0.001$ , respectively). Among proteinuric subjects, PCSK9 levels at baseline treatment were also higher in statin using compared to non-statin using subjects ( $P = 0.01$ ). In proteinuric subjects, PCSK9 was not associated with e-GFR ( $R = -0.083$ ,  $P = 0.62$ ) and borderline with BMI ( $R = 0.311$ ,  $P = 0.07$ ). In healthy subjects, PCSK9 was positively associated with BMI ( $R = 0.515$ ,  $P = 0.01$ ) and also unrelated to e-GFR ( $R = 0.178$ ,  $P = 0.34$ ). Multivariate regression analysis confirmed that PCSK9 levels were higher in statin using and non-statin using proteinuric subjects ( $\beta = 0.643$ ,  $P < 0.001$  and  $\beta = 0.243$ ,  $P = 0.04$ , respectively) compared to healthy subjects when taking between-group differences in BMI ( $\beta = 0.287$ ,  $P = 0.009$ ) and e-GFR ( $\beta = 0.175$ ,  $P = 0.19$ ) into account.

In the whole group of proteinuric subjects, proteinuria decreased from 1.9 [0.9–3.3] to 0.5 [0.3–1.1] g/day ( $P < 0.001$ ) upon



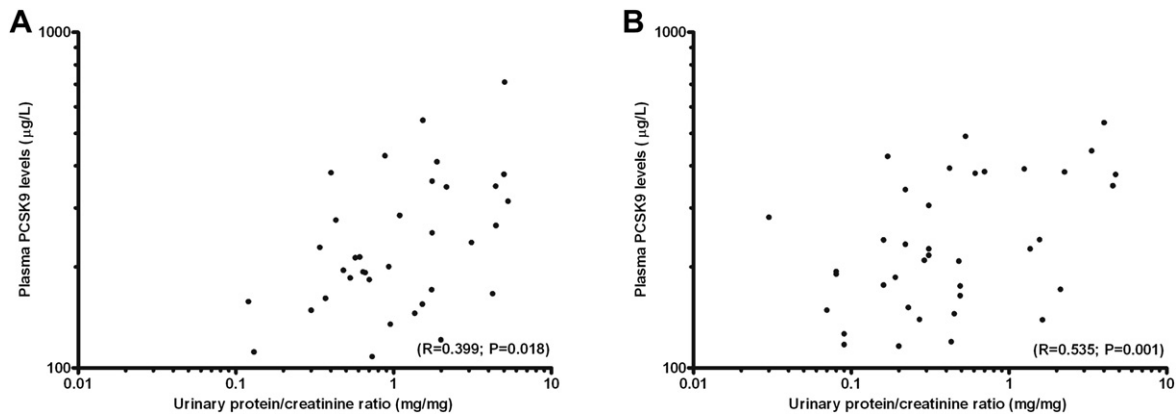
**Fig. 1.** Plasma PCSK9 levels in healthy subjects and proteinuric subjects at baseline treatment and maximal antiproteinuric treatment. Solid lines represent median plasma PCSK9 levels of healthy subjects and proteinuric subjects at baseline treatment and maximal antiproteinuric treatment. Statin and non-statin using proteinuric subjects are indicated as ■ and ■, respectively.

maximal antiproteinuric treatment. Creatinine-adjusted values of urinary protein excretion are shown in Table 1. Consequently, serum albumin increased from 39 [36–42] to 41 [37–44] g/L ( $P < 0.001$ ). In all proteinuric subjects combined, total cholesterol ( $P = 0.004$ ) and non-HDL cholesterol ( $P = 0.01$ ) decreased in response to maximal antiproteinuric treatment (Table 2A). The total cholesterol/HDL cholesterol ratio ( $P = 0.92$ ) and LDL cholesterol ( $P = 0.12$ ) did not significantly change, whereas there was a further drop in HDL cholesterol. However, in non-statin treated proteinuric subjects separately, not only total cholesterol but also LDL cholesterol and non-HDL cholesterol levels were decreased during maximal antiproteinuric treatment. Of further note, PCSK9 levels did not significantly respond to maximal antiproteinuric treatment (median change:  $-3$  [27–37] µg/L,  $P = 0.84$ ). Additionally, there was no difference in PCSK9 response between statin users compared with non-statin users (median change:  $-8$  [32–70] µg/L vs.  $-3$  [10–28] µg/L,  $P = 0.28$ ).

As shown in Fig. 2, PCSK9 levels were positively associated with the urinary protein/creatinine ratio at baseline treatment ( $R = 0.399$ ,  $P = 0.018$ ) and at maximal antiproteinuric treatment ( $R = 0.525$ ,  $P = 0.001$ ).

In healthy subjects, as well as in proteinuric subjects at baseline treatment, positive relationships of total cholesterol, non-HDL cholesterol, LDL cholesterol, triglycerides, total cholesterol/HDL cholesterol ratio and apoB with PCSK9 levels were found (Table 3). During maximal antiproteinuric treatment, comparable relationships between apoB-containing lipoproteins and PCSK9 were observed, although the relationship with LDL cholesterol ( $P = 0.274$ ) and the total cholesterol/HDL cholesterol ratio ( $P = 0.209$ ) did not reach significance (Table 3). Furthermore, individual changes in total cholesterol, non-HDL cholesterol and LDL cholesterol were associated positively with individual changes in PCSK9 levels, whereas such a trend was also found for apoB (Fig. 3). Changes in PCSK9 were not significantly related to changes in urinary protein/creatinine ratio ( $R = -0.284$ ,  $P = 0.11$ ).

The absolute responses in the total cholesterol/HDL cholesterol ratio were correlated with PCSK9 levels at baseline treatment ( $R = -0.428$ ;  $P = 0.007$ ). The individual responses in the total cholesterol/HDL cholesterol ratio were still predicted by PCSK9 levels at baseline treatment after adjustment for the baseline total cholesterol/HDL cholesterol ratio ( $\beta = 0.306$ ,  $P = 0.04$ ). Fig. 4 illustrates that higher PCSK9 levels at baseline treatment (divided in sex-specific tertiles) were associated with a more pronounced relative decrease in the total cholesterol/HDL cholesterol ratio in response to maximal antiproteinuric treatment.



**Fig. 2.** Scatter plots showing the univariate associations between urinary protein/creatinine ratio and plasma PCSK9 levels at baseline treatment (A) and maximal antiproteinuric treatment (B) in proteinuric subjects. Spearman's correlation coefficients are shown.

#### 4. Discussion

This study documents for the first time that plasma PCSK9 levels are elevated in subjects with glomerular proteinuria in respect to age- and sex-matched healthy subjects, also when taking concomitant statin treatment, renal function, and BMI into account. Furthermore, the extent to which PCSK9 was increased was proportional to the degree of proteinuria. PCSK9 levels did not decrease after maximal antiproteinuric treatment, but individual changes in total cholesterol, non-HDL cholesterol and LDL cholesterol were positively correlated with changes in PCSK9 in response to maximal antiproteinuric treatment. Taken together, the current observations are in line with the hypothesis that abnormalities in the PCSK9 pathway may contribute to the pathogenesis of glomerular proteinuria-associated alterations in apoB-containing lipoprotein metabolism.

The modest lipoprotein abnormalities in the proteinuric subjects at baseline treatment as observed in this report should be interpreted in context of current clinical practice to start early with antiproteinuric treatment, and to prescribe statin therapy to those with more severe hypercholesterolemia [4]. Our findings may, therefore, be regarded as preliminary and hypothesis-generating, and findings should be replicated in a larger cohort. The lack of inclusion of untreated patients in this study could be argued as

a limitation of our study. However, for medical ethical reasons it was considered not justified to stop antihypertensive and lipid-lowering therapy before entry in our trial. Thus, all participating proteinuric subjects received ACEi at baseline. Consequently, the degree of proteinuria at the baseline evaluation was only modest compared to previous reports in patients with comparable glomerular disorders [22,23]. Indeed, total cholesterol, LDL cholesterol, non-HDL cholesterol and the total cholesterol/HDL cholesterol ratio were much higher before initiating lipid-lowering treatment in those proteinuric subjects who received statin therapy at baseline. These circumstances largely explain why only the total cholesterol/HDL cholesterol ratio and triglycerides were higher in proteinuric subjects at baseline treatment compared to healthy subjects. Nonetheless, total cholesterol, LDL cholesterol and non-HDL cholesterol were lowered in response to maximal antiproteinuric treatment, particularly in non-statin treated proteinuric subjects.

Obviously, the elevated PCSK9 levels in proteinuric subjects reported here should also be interpreted against this context of concomitant lipid-lowering drug treatment as it is well documented that statins are able to upregulate PCSK9 via SREBP-2-mediated mechanisms [24,25]. Hence, we took statin therapy into account by separately comparing PCSK9 levels in statin using and non-statin using proteinuric subjects with their individually matched healthy subjects. PCSK9 levels were indeed higher in statin using proteinuric subjects compared to both matched healthy subjects and non-statin using proteinuric subjects. There are no published data concerning possible effects of renal impairment on PCSK9 regulation, but we did not find a relationship of PCSK9 levels with e-GFR in proteinuric and healthy subjects in this report. Nonetheless, it remains possible that severe CKD could affect the PCSK9 pathway. Moreover, the decrease in e-GFR as elicited by antiproteinuric treatment was reversible upon discontinuation of the low sodium diet and ARB (data not shown), and most probably reflects temporal reduction in intraglomerular pressure instead of natural progression of CKD. It is, therefore, very unlikely that progression of CKD interfered with lipoprotein or PCSK9 responses to treatment. Furthermore, the treatment sequence in our study was randomized. Since all proteinuric patients used ACEi therapy at baseline we cannot fully exclude a contribution of this treatment to PCSK9 elevations at baseline treatment, but plasma PCSK9 elevation due to ACEi treatment is counterintuitive in view of its proteinuria lowering effect. Of additional importance, PCSK9 relates positively to obesity [16], as confirmed here in healthy subjects. Multivariate analysis demonstrated that PCSK9 levels were still higher in proteinuric subjects compared to healthy subjects after controlling for concomitant

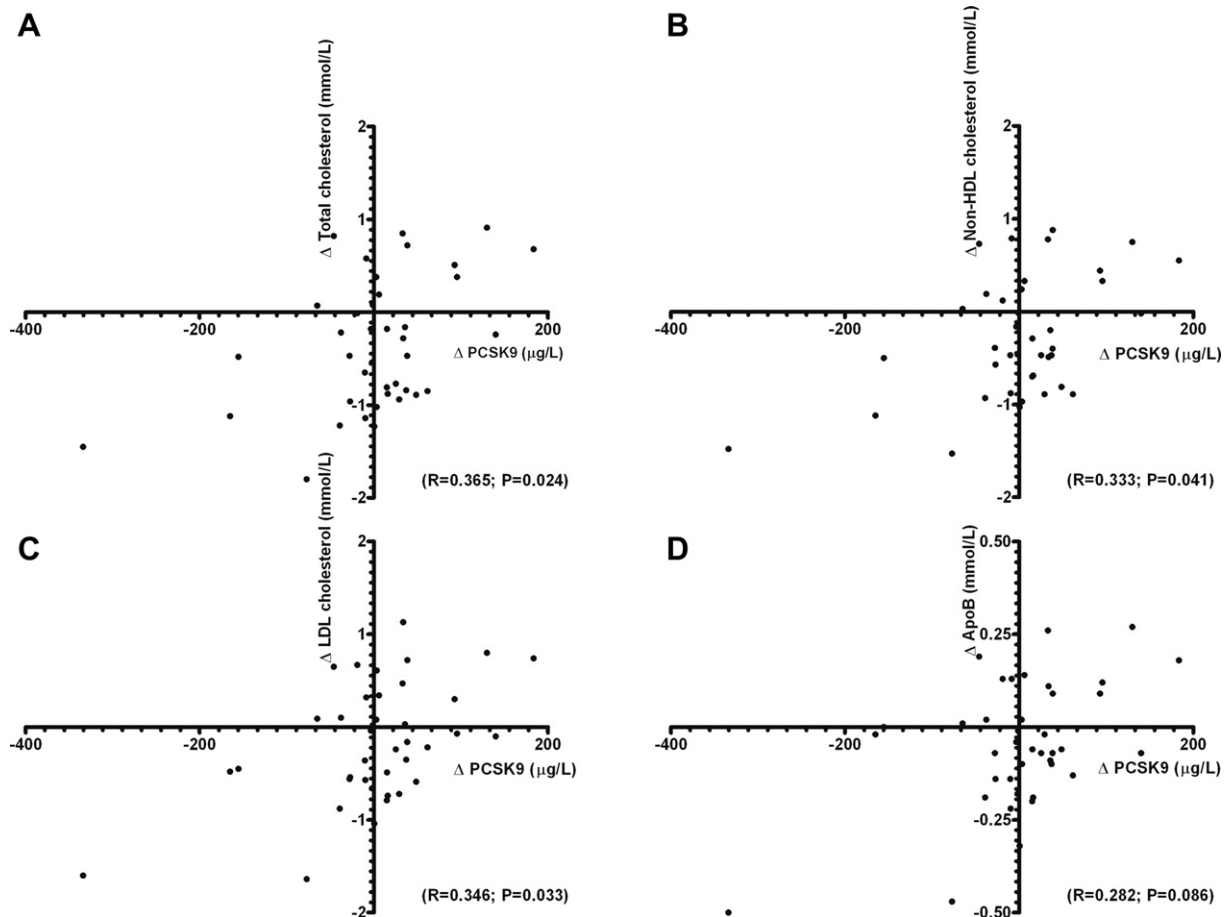
**Table 3**

Relationships of plasma lipoproteins with plasma PCSK9 levels in healthy subjects and proteinuric subjects at baseline treatment and maximal antiproteinuric treatment.

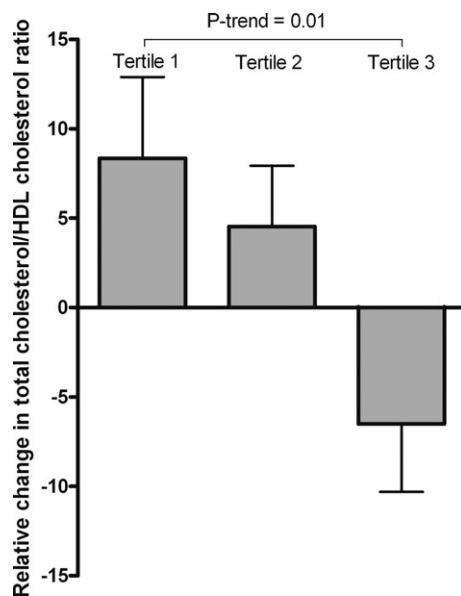
|   | Healthy subjects<br>(n = 39) | Proteinuric subjects (n = 39) |                                   |
|---|------------------------------|-------------------------------|-----------------------------------|
|   |                              | Baseline treatment            | Maximal antiproteinuric treatment |
|   | Correlation coefficients     | Correlation coefficients      | Correlation coefficients          |
| Total cholesterol (mmol/L)              | 0.574***                     | 0.468**                       | 0.467**                           |
| Non-HDL cholesterol (mmol/L)            | 0.675***                     | 0.446**                       | 0.458**                           |
| LDL cholesterol (mmol/L)                | 0.489**                      | 0.343*                        | 0.182                             |
| Triglycerides (mmol/L)                  | 0.738***                     | 0.396*                        | 0.546***                          |
| Total cholesterol/HDL cholesterol ratio | 0.738***                     | 0.305*                        | 0.208                             |
| ApoB (g/L)                              | 0.619***                     | 0.393*                        | 0.386*                            |

\* $P \leq 0.05$ . \*\* $P \leq 0.01$ . \*\*\* $P \leq 0.001$ .

Spearman's correlation coefficients are shown. LDL cholesterol: low density lipoprotein cholesterol; HDL cholesterol; high density lipoprotein cholesterol; ApoB: apolipoprotein B.



**Fig. 3.** Scatter plots showing the univariate associations between changes in PCSK9 and changes in total cholesterol (A), non-HDL cholesterol (B), LDL cholesterol (C) and ApoB (D) in proteinuric subjects. Spearman's correlation coefficients are shown.



**Fig. 4.** Relative change in total cholesterol/HDL cholesterol ratio from baseline treatment according to sex-specific tertiles of plasma PCSK9 levels at baseline treatment in proteinuric subjects. Ranges of sex-specific tertiles of plasma PCSK9 levels at baseline treatment were 108–171, 182–314, and 266–711 µg/L, respectively. Data are depicted as mean (SEM).

statin treatment and for differences in e-GFR and BMI. Thus, it can essentially be ruled out that our main finding with respect to PCSK9 elevations in proteinuric subjects is to a relevant extent confounded by moderate renal function impairment and/or by between-group differences in body weight excess. Importantly, PCSK9 levels were strongly associated with urinary protein excretion both before and after maximal antiproteinuric treatment. However, PCSK9 did not significantly decrease in response to intensified antiproteinuric treatment. Apparently, the amount of proteinuria reduction achieved in this trial may not have been sufficient to lower PCSK9. This would raise the possibility that even further lowering of proteinuria is required to affect circulating PCSK9 levels. It is also possible that the period of maximal antiproteinuric treatment was too short to elicit a PCSK9 response.

ApoB-containing lipoprotein levels were associated positively with PCSK9 levels in healthy subjects, which is in line with other reports [14–17,26], as well as in proteinuric subjects. More importantly, individual changes in apoB-containing lipoprotein levels in response to maximal antiproteinuric treatment were correlated positively with individual changes in PCSK9. Thus, although total cholesterol and non-HDL cholesterol decreased in response to maximal antiproteinuric therapy despite unchanged plasma PCSK9 on a group level, the present observations support the concept that changes in the PCSK9 pathway may contribute to abnormalities in apoB-containing lipoprotein metabolism in proteinuric subjects. In this regard it is also noteworthy that individual improvements in the total cholesterol/HDL cholesterol ratio after intensified antiproteinuric treatment were related to a higher baseline

treatment PCSK9 level. This relationship was still present after controlling for the total cholesterol/HDL cholesterol ratio at baseline. This finding would thus raise the possibility that the more pronounced the PCSK9-related changes in the LDL-receptor pathway the more responsive the changes in the lipoprotein cholesterol ratio in response to antiproteinuric treatment. Moreover, this observation suggests that measurement of PCSK9 may be clinically relevant in predicting lipoprotein responses after intensified antiproteinuric treatment. In this context, it is important that higher plasma PCSK9 levels predict recurrent cardiovascular events in high risk subjects during statin treatment [21].

The precise mechanisms whereby the severity of urinary protein loss may lead to alterations in the PCSK9 pathway are unknown at present. Hepatocellular LDL-receptor protein mass rather than its messenger RNA expression has been documented to be deficient in experimental nephrosis [8]. It is, therefore, likely that post-translational abnormalities in LDL-receptor abundance explain at least in part atherogenic lipoprotein changes associated with proteinuria. Since PCSK9 directs the LDL-receptor towards intracellular degradation (as under viewed in [13]), the results in experimental nephrosis [8] agree with the concept that activation of the PCSK9 pathway may contribute to proteinuria-associated down regulation of hepatic LDL expression.

Attempts to improve cardiovascular outcome in patients with renal disease have been to some extent disappointing [27,28], although a modest reduction in incidence of atherosclerotic events was found in the SHARP trial [29]. Interestingly, a recent phase-1 trial in healthy subjects and in subjects with hypercholesterolemia revealed that inhibition of PCSK9 by monoclonal antibodies decreased LDL cholesterol by 41–65%, irrespective of concomitant statin treatment [30]. In view of our current findings which favor the hypothesis that PCSK9 may contribute to atherogenic lipoprotein abnormalities in proteinuric subjects, it is tempting to propose that proteinuric subjects represents a relevant patient category for PCSK9 inhibition in the future.

In conclusion, plasma PCSK9 levels are elevated in subjects with glomerular proteinuria, proportional to the degree of proteinuria. This study fits into the concept that the PCSK9 pathway may play a role in atherogenic lipoprotein changes in human proteinuria. Inhibition of the PCSK9 pathway could become an important treatment target in proteinuric patients.

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## Disclosures

None to declare.

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