# Proteinuria converts hepatic heparan sulfate to an effective proprotein convertase subtilisin kexin type 9 enzyme binding partner

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# Proteinuria converts hepatic heparan sulfate to an effective proprotein convertase subtilisin kexin type 9 enzyme binding partner



OPEN

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**Hepatic uptake of triglyceride-rich remnant lipoproteins is mediated by the low-density lipoprotein receptor, a low-density lipoprotein receptor related protein and the heparan sulfate proteoglycan, syndecan-1. Heparan sulfate proteoglycan also mediates low-density lipoprotein receptor degradation by a regulator of cholesterol homeostasis, proprotein convertase subtilisin kexin type 9 (PCSK9), thereby hampering triglyceride-rich remnant lipoproteins uptake. In this study, we investigated the effects of proteinuria on PCSK9, hepatic heparan sulfate proteoglycan and plasma triglyceride-rich remnant lipoproteins. Adriamycin-injected rats developed proteinuria, elevated triglycerides and total cholesterol (all significantly increased). Proteinuria associated with triglycerides and total cholesterol and serum PCSK9 (all significant associations) without loss of the low-density lipoprotein receptor as evidenced by immunofluorescence staining and western blotting. In proteinuric rats, PCSK9 accumulated in sinusoids, whereas in control rats PCSK9 was localized in the cytoplasm of hepatocytes. Molecular profiling revealed that the heparan sulfate side chains of heparan sulfate proteoglycan to be hypersulfated in proteinuric rats. Competition assays revealed sulfation to be a major determinant for PCSK9 binding. PCSK9 partly colocalized with hypersulfated heparan sulfate in proteinuric rats, but not in control rats. Hence, proteinuria induces hypersulfated hepatic heparan sulfate proteoglycans, increasing their affinity to PCSK9. This might impair hepatic triglyceride-rich remnant lipoproteins uptake, causing proteinuria-associated dyslipidemia. Thus, our study reveals PCSK9/heparan sulfate may be a novel target to control dyslipidemia.**

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## Translational Statement

Cardiovascular complications due to dyslipidemia are of concern in chronic kidney disease. Herein, we discovered that proteinuria induces hypersulfation of hepatic heparan sulfate proteoglycans, increasing their affinity to proprotein convertase subtilisin kexin type 9 (PCSK9) and causing sinusoidal accumulation of PCSK9. These changes were associated with elevated plasma triglycerides and total cholesterol, without affecting low-density lipoprotein receptor expression. These data indicate a novel mechanism behind dyslipidemia in chronic kidney disease. At present, where current cholesterol-lowering strategies to treat dyslipidemia in chronic kidney disease are still insufficient and novel PCSK9 inhibitors are being introduced, our study opens new venues for future development of cost-effective heparin mimetics as PCSK9 inhibitors.

Chronic kidney disease (CKD) is a global health problem affecting >10% of the people worldwide.[1] Cardiovascular diseases (CVDs) are the leading cause of mortality in CKD.[2,3] Incidence of coronary heart disease is 40% in end-stage CKD, and CVD-related mortality is 10 to 30 times higher than in the general population of same sex, age, and race.[3,4] Higher risk of CVD in CKD is largely due to anomalies in lipoprotein metabolism, represented by high plasma triglycerides (TGs), total cholesterol (TC), low-density lipoprotein cholesterol (LDLc), lipoprotein-a, and low high-density lipoprotein cholesterol (HDLc) levels. The severity of dyslipidemia in CKD depends on level of proteinuria[5,6] and stage of kidney disease.[2,7]

Although CKD patients without proteinuria display high TC, LDLc, and TGs, CKD patients with proteinuria have worse dyslipidemic phenotype than general population and non-nephrotic CKD population.[2,5,7–9] Similarly, reducing proteinuria lowers TC and TGs, proportionally, irrespective of the type of intervention,[10–12] indicating proteinuria as an independent risk factor for CVD-related mortality.[12]

Pathophysiology behind CKD-dyslipidemia includes deficiencies of lipoprotein lipase, hepatic lipase, and lecithin–cholesterol acyltransferase, increased angiopoietin-like protein 4, and increased endogenous cholesterol production. Also, the reduced clearance of triglyceride-rich remnant lipoproteins due to hepatic loss of syndecan-1, low-density lipoprotein receptor (LDLR)–related protein (LRP-1), and/or LDLR is reported.[12–17]

Heparan sulfate (HS) proteoglycans (HSPGs) present in the basal cell membranes of hepatocytes (mainly syndecan-1) and partake in lipoprotein uptake and clearance, independent of LDLR and LRP-1.[18–20] All these receptors capture triglyceride-rich remnant lipoproteins from space of Disse and get internalized. Inside the hepatocytes, they release the lipoproteins that are eventually hydrolyzed by lysosomal acid lipase, and cholesterol is taken up by the cells. The receptors then recycle back to the surface to repeat the process.[21–24] Hepatic HS contains highly sulfated domains within shorter chains compared with other tissues.[20] Highly sulfated motifs are clustered distal from the protein core, allowing interaction and binding to triglyceride-rich remnant lipoproteins.[20] Changes in the structure/sulfation of HS chains affect the functionality of HSPGs.[25–27] We previously showed loss of hepatic HSPGs to be associated with increased plasma TG in renal transplant recipients.[28] This might suggest reduced HSPG-mediated TRL clearance as a cause of dyslipidemia in renal transplantation. We also showed hypersulfation of renal HS in proteinuric renal diseases[29]; however, we never investigated the effects of proteinuria on hepatic HS.

Recently, Gustafsen *et al.* reported hepatic HSPGs as liver-specific coreceptors for proprotein convertase subtilisin kexin type 9 (PCSK9). Through the HS chains, HSPGs interact with prodomain of PCSK9 and present PCSK9 to LDLR.[30] Several studies have reported increase of plasma PCSK9 in CKD and a strong positive association with proteinuria.[31,32] Moreover, Haas *et al.* reported a reduction in PCSK9 levels in nephrotic patients after effective treatment of nephrotic syndrome.[33] These studies suggest that PCSK9-mediated LDLR degradation is increased in CKD, hampering the normal lipoprotein clearance.

In the current study, we have studied the effects of proteinuria on hepatic HSPGs, PCSK9, and their effects on lipoprotein metabolism using Adriamycin (doxorubicin)–induced proteinuria model. This model is a pure proteinuria model (due to the damaging effect of Adriamycin on glomerular podocytes) without loss of renal function (glomerular filtration rate).[34] We observed that proteinuria induces dyslipidemia and hypersulfation of hepatic HSPGs, ultimately affecting HSPG-PCSK9 interaction and the localization of PCSK9 in the livers. Based on these data, we propose a novel mechanism and treatment strategy in proteinuria-induced dyslipidemia.

## METHODS

### Animals and treatments

Proteinuria was induced in 3-month-old male Wistar rats (weighing 180–200 g) by single injection of Adriamycin (1.8 mg/kg body weight), as described by Yazdani *et al.* (n = 8).[35] Healthy, age- and sex-matched rats with saline injection served as controls (n = 6). At week 12, organs were harvested after saline perfusion and preserved in liquid nitrogen. Creatinine in plasma and urine was measured by an enzymatic UV assay (Roche Modular P, Mannheim, Germany), and total urinary protein was measured by turbidimetric assay (Roche Modular P). Experimental procedures were performed according to the national guidelines for the care and use of laboratory animals,and approved by the local Animal Ethics Committee of the University of Groningen.

### Plasma cholesterol profiling

Rat plasma samples were fractionated by fast protein liquid chromatography, as previously described by Bartuzi *et al.*[22]

### Immunofluorescence staining

Hepatic lipoprotein receptors, PSCK9 and HS, were visualized by (confocal) immunofluorescence. Details on tissues, antibodies, and quantification of the stainings are given in Supplementary Table S2.

### Western blotting

Liver homogenates from control rats and Adriamycin-treated animals were used for immunoblotting, as described by Fedoseienko *et al.*[21] Antibodies used were as follows: rabbit polyclonal anti-LDLR (1:300 in phosphate-buffered saline/1% bovine serum albumin; Pab8804; Abnova, Taipei City, Taiwan); rabbit monoclonal antibody (mAb) anti–LRP-1 (1:7500 in phosphate-buffered saline/1% bovine serum albumin; Abcam 92544, Cambridge, UK); and rabbit anti-mouse PCSK9 (552C; 1:300 in phosphate-buffered saline/1% bovine serum albumin), which was kindly provided by Jayson d'Horton (University of Texas Southwestern Medical Center, Dallas, TX).[36,37] Goat anti-rabbit IgG horseradish peroxidase (DAKO, Heverlee, Belgium) was used as a secondary antibody.

**Table 1 | Clinical characteristics of healthy controls and Adriamycin rats at 12 weeks**

| Parameters | Controls (n = 6) | Adriamycin (n = 8) | P value |
|---|---|---|---|
| Body weight, g | 472 ± 12 | 446 ± 9 | NS |
| Blood pressure, mm Hg | | | |
| Systolic blood pressure | 144 ± 7 | 137 ± 5 | NS |
| Diastolic blood pressure | 87 ± 7 | 89 ± 3 | NS |
| Mean blood pressure | 108 ± 8 | 106 ± 4 | NS |
| Renal function | | | |
| Plasma creatinine, μmol/l | 26 ± 1 | 26 ± 1 | NS |
| Creatinine clearance, ml/min | 4.8 ± 0.2 | 4.8 ± 0.2 | NS |
| Proteinuria, mg/24 h | 50 ± 16 | 343 ± 63 | [a] |
| Plasma lipid parameters, mmol/l | | | |
| Triglycerides | 1.49 ± 0.20 | 4.74 ± 1.29 | [a] |
| Total cholesterol | 1.58 ± 0.09 | 4.00 ± 0.76 | [a] |

NS, no significant difference.
[a]$P < 0.005$.
Data represent mean and SEM.

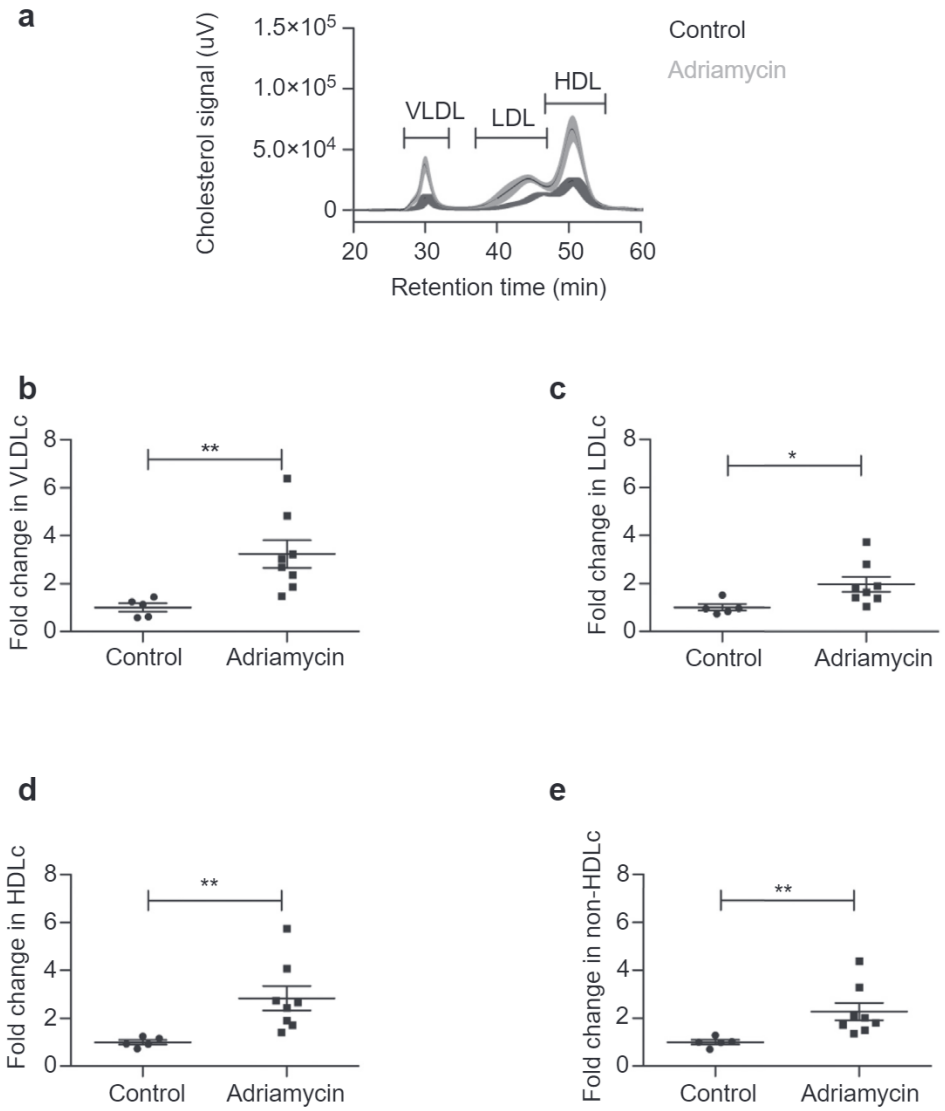


**Figure 1 | Cholesterol profiling by fast-performance liquid chromatography (FPLC) in control rats and Adriamycin-treated rats.** (**a**) FPLC profiles for plasma cholesterol of individual rats for control rats (n = 5) and Adriamycin-treated rats (n = 8). The dark line indicates the mean, and the light shades indicate SEM. (**b**–**e**) Fold increase in very-low-density lipoprotein (VLDL) cholesterol (VLDLc; **b**), in low-density lipoprotein (LDL) cholesterol (LDLc; **c**), in high-density lipoprotein (HDL) cholesterol (HDLc; **d**), and in non-HDL cholesterol (non-HDLc; **e**) in control rats and Adriamycin-treated rats. Non-HDLc was measured as the sum of VLDLc and LDLc. Data shown as mean ± SEM. $^{*}P < 0.05$, $^{**}P < 0.01$.

### Dot blot assay

Proteins were isolated from liver cryosections of control rats and Adriamycin-treated rats and measured as described by Fedoseienko *et al.*[21] A total of 100 µl of samples at concentration 0.625 µg/100 µL of total protein was blotted on a polyvinylidene difluoride membrane with the aid of a BIODOT (BioRad, Hercules, CA) device, and dot blot was done as previously described by Adepu *et al.*[28] As a positive control, recombinant syndecan-1 was spotted on the membrane. Wells without any spotted sample, but incubated with primary and secondary antibodies, served as negative controls.

### RNA isolation, cDNA synthesis, and quantitative reverse transcriptase–polymerase chain reaction

Total mRNA was isolated from liver tissues of control rats and Adriamycin-treated group using RNeasy mini kit (Favorgen Biotech Corp., Ping-Tung, Taiwan), as described by Yazdani *et al.*[35] Housekeeping gene was endogenous *Gapdh*. Transcripts for *Ldlr*, *Pcsk9*, *Lrp-1*, *Syndecan-1*, *Sulf 2*, enzymes involved in HS synthesis and degradation, and enzymes involved in cholesterol biosynthesis were measured (primers in Supplementary Table S1). All primers were purchased from Sigma Aldrich (St. Louis, MO), except *Syndecan-1*, *Ldlr*, and *Sulf2*, which were from Qiagen (Hilden, Germany). The cycle procedure was as described previously by Yazdani *et al.*[35] Negative controls are included using the samples without cDNA (polymerase chain reaction–grade water and mRNA sample mix where no reverse transcriptase was added while making cDNA). Measurements were done in triplicate.

### Extraction and purification of HS from liver samples

From each rat, ~30 mg frozen liver tissue was collected. Five liver samples per group were pooled, resulting in 2 pooled samples (control rats and Adriamycin-treated rats). Extraction and

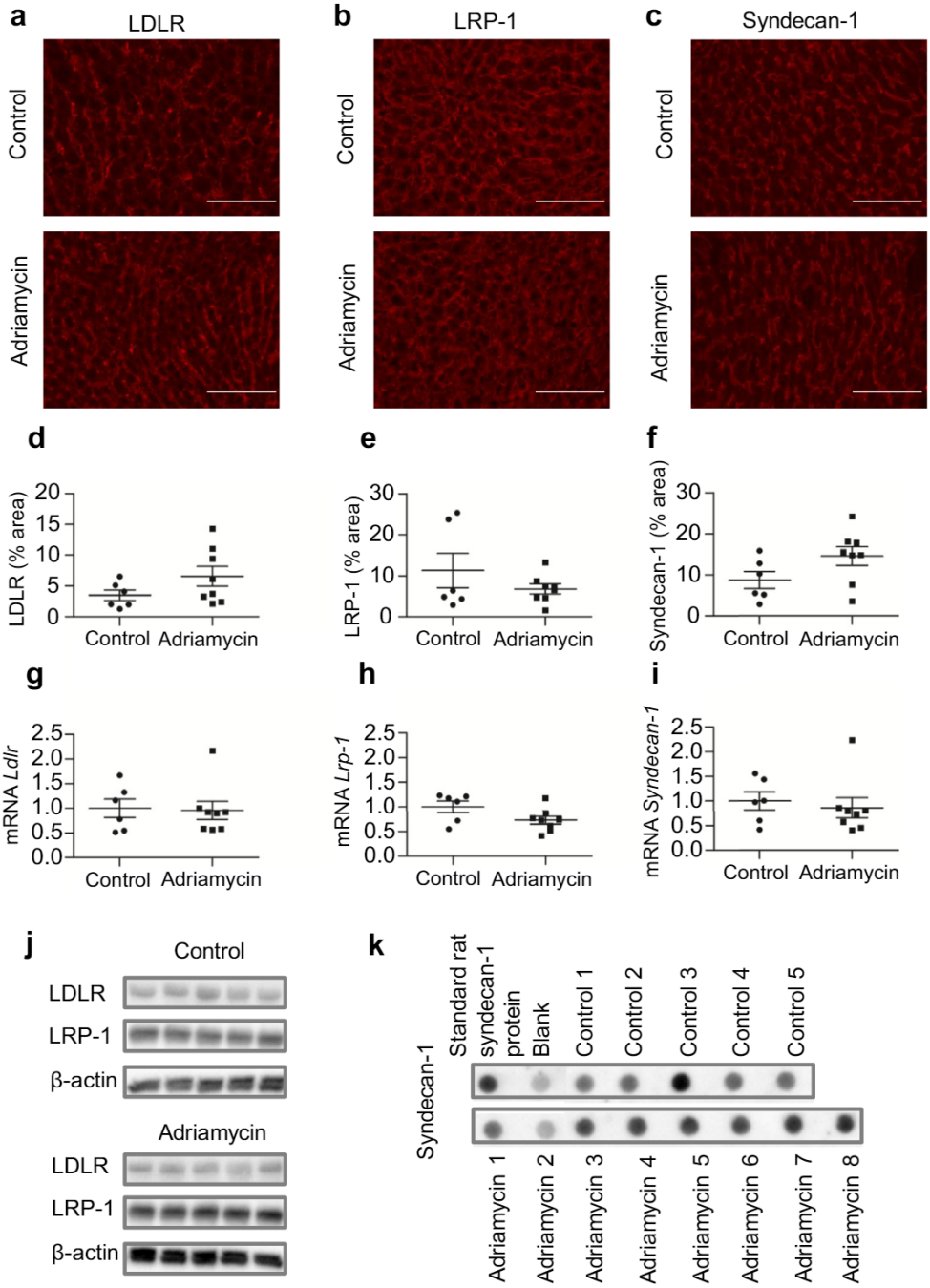


**Figure 2| Expression of hepatic lipoprotein receptors low-density lipoprotein receptor (LDLR), LDLR-related protein (LRP-1), and syndecan-1 in control rats and Adriamycin-treated rats.** (**a**–**c**) Immunofluorescence staining of hepatic LDLR, LRP-1, and syndecan-1. Bars = 100 μm. (**d**–**f**) Quantification of immunofluorescence staining representing surface expression of hepatic LDLR, LRP-1, and syndecan-1. (**g**–**i**) mRNA expression of hepatic *Ldlr*, *Lrp-1*, and *Syndecan-1* relative to *Gapdh*. (**j**) Measurement of LDLR and LRP-1 protein by Western blot. (**k**) Measurement of syndecan-1 protein by dot blot. No significant differences were found between control rats and Adriamycin-treated rats. Data shown as mean ± SEM. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

purification of HS was performed as described previously in Ledin *et al.*[38]

### HS disaccharide analysis

Disaccharide analysis of HS was performed by reverse-phase ion-pair high-performance liquid chromatography, as described by Hijmans *et al.*[27] Disaccharide isolation and analyses of each pool were performed twice in triplicate.

### PCSK9 enzyme-linked immunosorbent assay

PCSK9 plasma levels in control rats and Adriamycin-treated rats were determined using rat PCSK9 enzyme-linked immunosorbent assay kit (SEK80005; Sino Biological Inc., Beijing, China).

### PCSK9 competition assay

The ability of heparin/nonanticoagulant heparin derivatives to compete with heparin-albumin for binding to PCSK9 was evaluated

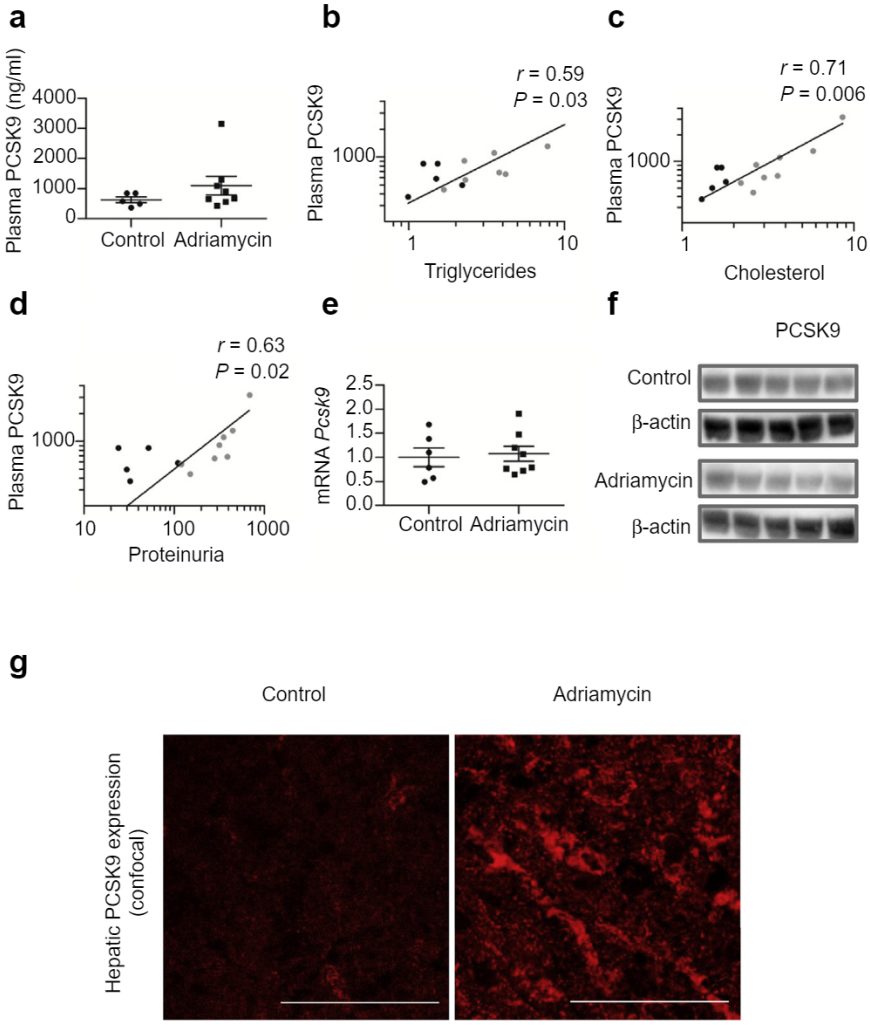


**Figure 3 | Expression of proprotein convertase subtilisin kexin type 9 (PCSK9) in serum and livers of control rats and Adriamycin-treated rats.** (**a**) Serum PCSK9 levels (ng/ml). (**b**–**d**) Correlation of serum PCSK9 (ng/ml) with serum triglycerides (mmol/L), serum cholesterol (mmol/L), and proteinuria (mg/24 h). (**e**) Measurement of mRNA expression of hepatic *Pcsk9* by quantitative reverse transcriptase–polymerase chain reaction. (**f**) Measurement of total protein of PCSK9 by Western blot. (**g**) Confocal immunofluorescence images representing localization of hepatic PCSK9. Bars = 50 μm. Data shown as mean ± SEM. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

by enzyme-linked immunosorbent assay. Details can be found in Supplementary Methods S1.

### Statistical analysis

Analyses were performed using GraphPad version 5 (La Jolla, CA). Mann-Whitney *U*-test was used for statistical significance between the groups. Data are given as means ± SEM. Associations were analyzed by nonparametric Spearman correlation. $P < 0.05$ was taken as statistically significant.

## RESULTS

### Clinical parameters in a rat model for proteinuria

Clinical parameters in control rats and Adriamycin-treated rats were assessed 12 weeks after saline or Adriamycin injection (Table 1). Adriamycin-treated rats developed proteinuria, without loss of renal function. They developed significant increase in plasma TG and TC (very-low-density lipoprotein cholesterol [VLDLc], LDLc, and HDLc) levels and in non-HDLc (VLDLc + LDLc) levels (Figure 1). Proteinuria was positively associated with plasma TG ($r = 0.86$; $P = 0.0003$) and TC levels ($r = 0.96$; $P < 0.0001$). Overall, herein, we show that proteinuria induced by Adriamycin causes hyperlipidemia in rats.

### Proteinuria does not affect the protein expression of hepatic lipoprotein receptors

Because LDLR, LRP-1, and syndecan-1 are the major hepatic receptors for VLDL and LDL, we studied their expression by immunofluorescence staining, quantitative reverse transcriptase–polymerase chain reaction, and Western blot or dot blotting (Figure 2). We observed sinusoidal staining pattern, because all 3 receptors are present at the basolateral membranes of hepatocytes. No differences were observed in staining intensity or distribution (Figure 2a–f) and mRNA expression (Figure 2g–i) of all the receptors between the groups. Western

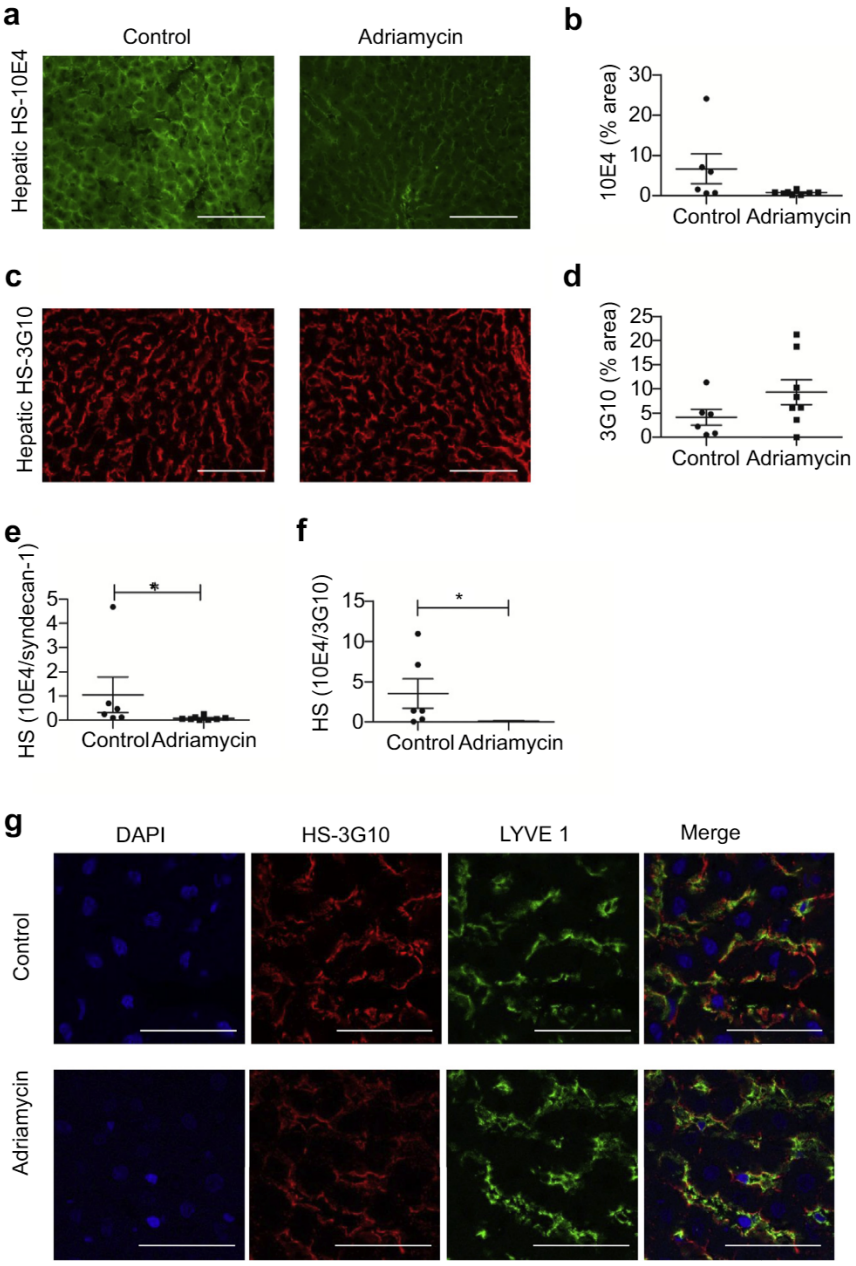


**Figure 4 | Expression of hepatic heparan sulfates (HSs) by anti-HS monoclonal antibodies (mAbs) 10E4 and 3G10 in control rats and Adriamycin-treated rats.** (**a**) Immunofluorescence staining representing surface expression of HS by anti-HS mAb 10E4. (**b**) Quantification of HS by anti-HS mAb 10E4. (**c**) Immunofluorescence staining representing surface expression of HS by anti-HS mAb 3G10. Bars = 100 μm. (**d**) Quantification of HS by anti-HS mAb 3G10. (**e**) The relative expression of 10E4 to syndecan-1 core protein. (**f**) The relative expression of 10E4 to 3G10. (**g**) Confocal immunofluorescence images of hepatic HS using anti-HS mAb 3G10 with sinusoidal endothelial marker lymphatic vessel endothelial hyaluronan receptor 1. 4′,6-Diamidino-2-phenylindole (DAPI) represents nuclear staining. Bars = 50 μm. Data shown as mean ± SEM. *$P < 0.05$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

blot and dot blot analysis confirmed no changes in total protein expression of all receptors (Figure 2j and k) between the groups. These data indicate that proteinuria does not affect expression of hepatic lipoprotein receptors.

### Proteinuria does not affect endogenous cholesterol production

Despite no changes in hepatic lipoprotein receptors, Adriamycin-treated rats showed significant increase in serum TG and TC (VLDLc, LDLc, and HDLc) levels and in non-HDLc (VLDLc + LDLc) levels. Therefore, next, we investigated the expression of genes involved in endogenous cholesterol synthesis. We observed no significant differences in mRNA expression of *Acat1*, *Acat2*, *Mvd*, *Hmgcr*, *Hmgcs1*, *Mvk*, and *Sqle* (Supplementary Table S1) in between the groups. This excludes increased endogenous cholesterol production as a cause behind nephrotic dyslipidemia.

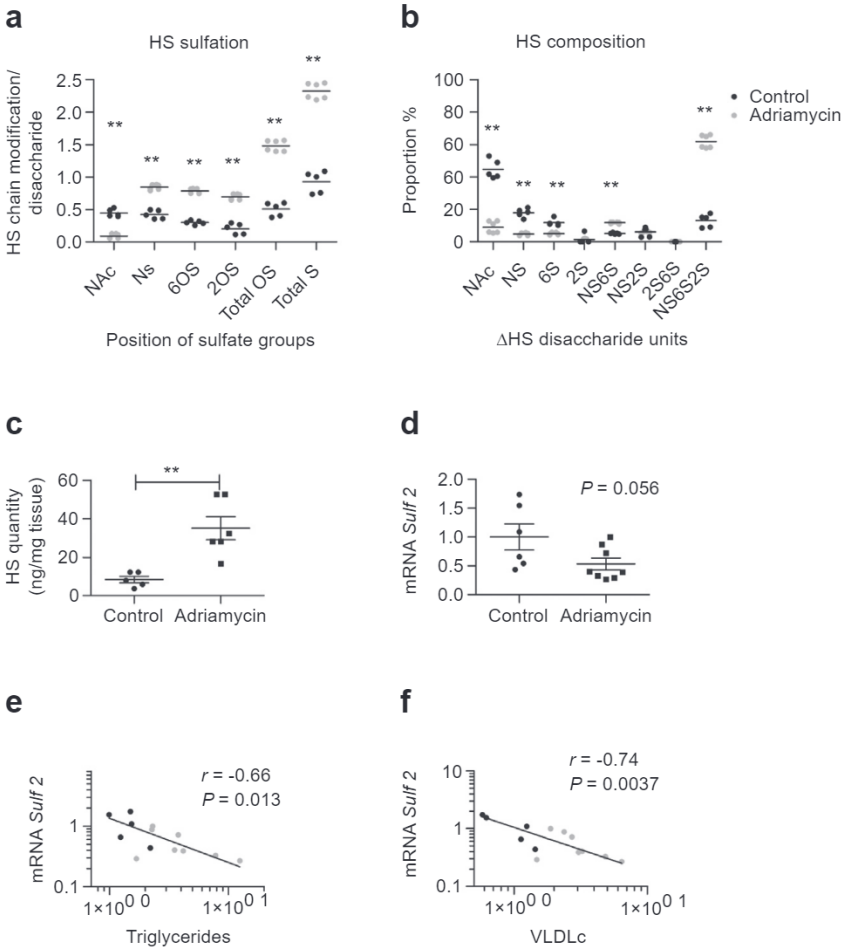


**Figure 5 | Molecular analysis of hepatic heparan sulfate (HS) in control rats and Adriamycin-treated rats.** (**a,b**) Hepatic HS disaccharide analysis by high-performance liquid chromatography of control rats and Adriamycin-treated rats. (**c**) Total quantity of hepatic HS of control rats and Adriamycin-treated rats. (**d**) Hepatic *Sulf-2* mRNA relative to *Gapdh* of Adriamycin-treated group compared with control rats. (**e,f**) Univariate correlation analysis of mRNA *Sulf-2* with triglycerides and very-low-density lipoprotein cholesterol (VLDLc). $^{**}P < 0.01$. 2OS, 2-O-sulfated uronic acid; 2S, 2-sulfated uronic acid; 2S6S, 2-O-sulfated uronic acid, 6-O-sulfated glucosamine; 6OS, 6-O-sulfated glucosamine; 6S, 6-O-sulfated glucosamine; NAc, N-acetylated glucosamine; NS, N-sulfated glucosamine; NS2S, N-sulfated glucosamine, 2-O-sulfated uronic acid; NS6S, N-sulfated, 6-O-sulfated glucosamine; NS6S2S, N-sulfated, 6-O-sulfated glucosamine, 2-O-sulfated uronic acid; Total OS, total O-sulfation; Total S, total sulfation.

### The localization of PCSK9 in rat livers is affected by proteinuria

Because PCSK9 hampers hepatic lipoprotein uptake by LDLR degradation, we measured plasma PCSK9 levels. No significant difference was observed (Figure 3a). Interestingly, however, plasma PCSK9 positively associated with plasma TG ($r = 0.59$; $P = 0.03$), TC ($r = 0.71$; $P = 0.0061$), and proteinuria ($r = 0.63$; $P = 0.022$), suggesting PCSK9 might be involved in proteinuric dyslipidemia (Figure 3b–d). Proteinuria did not affect the mRNA and protein levels of hepatic PCSK9 (Figure 3e and f). We also evaluated hepatic localization of PCSK9 in both groups by confocal immunofluorescence microscopy (Figure 3g). In proteinuric rats, PCSK9 was clustered and condensed in sinusoids compared with the finer granular cytoplasmic staining in control rats, indicating that proteinuria induces changes in the distribution of hepatic PCSK9.

### Proteinuria increases the level of sulfation of hepatic HS

Because both PCSK9 and lipoproteins can bind to HS, next we investigated the effects of proteinuria on the structure of hepatic HS. First, we performed immunofluorescence staining using anti-HS mAbs 10E4 and 3G10. As reported before, anti-HS mAb 10E4 binds to HS domains having mixed N-acetylated and N-sulfated disaccharide units.[39] The mAb 3G10 reacts with HS neoepitopes containing unsaturated uronic acid residues, generated by digesting HS with heparitinase I.[40] Both mAbs 10E4 and 3G10 marked the sinusoids of the liver. No significant changes in intensity and localization of 10E4 and 3G10 epitopes were observed between the groups, although 10E4 tended to be reduced and 3G10 tended to be increased in proteinuric rats (Figure 4a–d). Because reduced intensity of 10E4 HS might be the result of either lower expression levels of HSPGs, such as syndecan-1, or a lower total amount of HS (3G10 stains all HS chains), we calculated

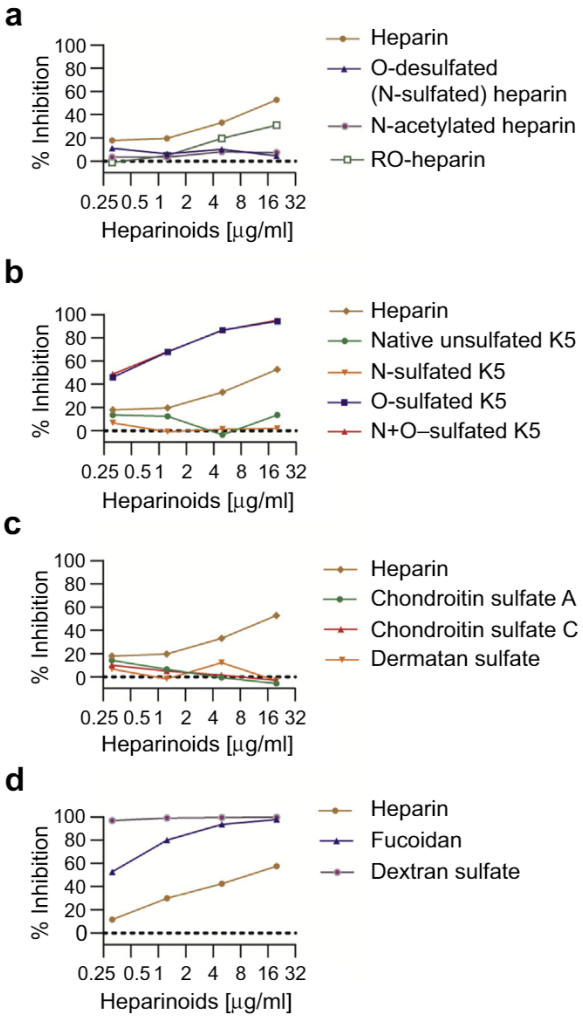


**Figure 6| Heparinoid binding to proprotein convertase subtilisin kexin type 9 (PCSK9) is dependent on the degree of sulfation.** (**a**) Unfractionated heparin and nonanticoagulant RO-heparin with the same degree of sulfation were effective in PCSK9 binding, whereas N-acetylated and O-desulfated heparins resulted in a loss of PCSK9 binding. (**b**) K5 polysaccharide preparations (with the same backbone structure as heparan sulfate and heparin): Native K5 and partial N-sulfated K5 could not compete with heparin-albumin binding of PCSK9, whereas both O-sulfated K5 and N + O–sulfated K5 equally competed for PCSK9 binding efficiently. (**c**) Chondroitin sulfate A and C and dermatan sulfate showed no competition for PCSK9 binding. (**d**) Highly sulfated polysaccharides (fucoidan and dextran sulfate) are also effective PCSK9-binding competitors. Data are expressed as percentage of inhibition of PCSK9 binding to immobilized PCSK9.

the expression of 10E4 relative to both syndecan-1 core protein and to 3G10. The relative expression of 10E4 to both syndecan-1 core protein and to 3G10 was significantly reduced in proteinuric rats (Figure 4e and f), indicating that the lower 10E4 staining is not due to lower syndecan-1 or lower HS expression levels, but rather to a change in HS sulfation, likely hypersulfation. Confocal double staining of 3G10/HS with sinusoidal endothelial marker lymphatic vessel endothelial hyaluronan receptor 1 showed HS on the outer margins of the sinusoids, most likely the basolateral membrane of the hepatocytes, where also syndecan-1 is localized (Figure 4g). These data indicate that proteinuric condition changed HS structure, but not its distribution, in hepatic tissues.

Second, we investigated the structural changes in hepatic HS chains by disaccharide analysis. This analysis revealed an unusual high degree of sulfation of HS in proteinuric rats, represented by increased N-sulfation, 6-O-sulfation, 2-O-sulfation, total O-sulfation, and total (N + O) sulfation (Figure 5a), resulting in ~2-fold increase in NS6S and ~5-fold increase in NS6S2S compared with control rats (Figure 5b). We also measured uronic acid content in isolated HS fractions. As represented in Figure 5c, total amount of HS was found to be significantly higher in Adriamycin-treated group. Together with unchanged syndecan-1 and 3G10-HS expression, our data indicate that under proteinuric conditions, hepatic HS chains are hypersulfated and probably longer as well.

Third, we evaluated the mRNA expression of most crucial enzymes involved in the synthesis, modification, and degradation of HS side chains. No significant differences were observed in the expression of chain polymerization and sulfation enzymes (Supplementary Table S1). However, an enzyme that selectively removes 6-O-sulfate groups from HS, known as HS-6-O-sulfatase-2 (SULF2),[41] was borderline reduced ($P = 0.059$) in Adriamycin-treated rats (Figure 5d). Increase in the NS2S6S (Figure 5b), the substrate for SULF2, supports reduction in mRNA *Sulf2*. This could explain the increased 6-O sulfate groups in hepatic HS chains in proteinuric animals. Interestingly, *Sulf2* mRNA expression was negatively associated with plasma TG ($r = –0.66$; $P = 0.013$), VLDLc ($r = –0.74$; $P = 0.0037$) (Figure 5e and f), TC ($r = –0.64$; $P = 0.018$), and LDLc ($r = –0.62$; $P = 0.02$) (not shown). Altogether, these data indicate that proteinuria affects the structure of hepatic HS, making them hypersulfated, and likely elongated.

### Binding affinity of PCSK9 to heparinoids depends on degree of sulfation

It has recently been shown that PCSK9 binds to HSPGs.[30] Therefore, we investigated if the sulfation degree of HS side chains influences HS-PCSK9 interaction. We conducted an HS-PCSK9 binding assay, and different heparinoids were evaluated for competition of PCSK9 interaction with immobilized heparin-albumin. Next to heparin, we tested 2 unfractionated nonanticoagulant heparinoids: RO-heparin and N-desulfated, reacetylated heparin. Both heparins have the same disaccharide building block composition and molecular weight (around 15 kDa), but vary in the degree of sulfation (RO-heparin having ~2.5 sulfate groups/disaccharide, similar to regular heparin, whereas N-desulfated/N-reacetylated heparin contains 1.5 sulfate groups/disaccharide). As shown in Figure 6a, we observed that RO-heparin exhibited a concentration-dependent ability to compete for PCSK9 binding to immobilized heparin-albumin, whereas N-acetyl heparin clearly lost all

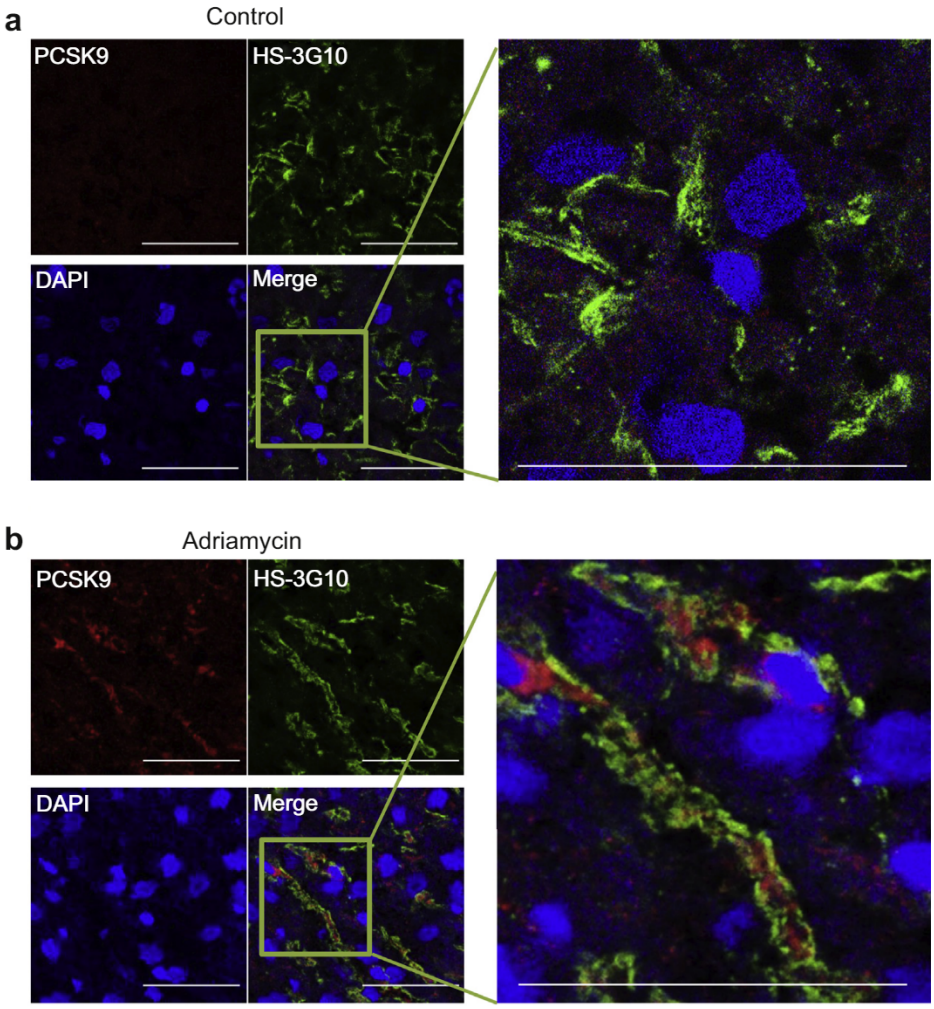


**Figure 7 | Confocal immunofluorescence double stainings of proprotein convertase subtilisin kexin type 9 (PCSK9) with anti–heparan sulfate (HS) monoclonal antibody 3G10.** (**a,b**) PCSK9 partly colocalizes with HS at the hepatic sinusoids in Adriamycin-treated rats, which, however, is absent in control rats. Bars = 50 μm. DAPI, 4′,6-diamidino-2-phenylindole. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

capability to inhibit the PCSK9-heparin interaction. These data show that the level of sulfation dictates PCSK9 binding. We also tested O-desulfated heparin (thus, only N-sulfated, 1.0 sulfate groups/disaccharide, same chain length as regular heparin). O-desulfated heparin showed no competition, further strongly supporting that sulfation is important for PCSK9 binding.

In addition, we also compared *Escherichia coli*–derived K5 polysaccharide derivatives, next to heparin. K5 polysaccharide contains the identical backbone structure as heparosan, the precursor molecule of HS and heparin (repeating dimer of D-glucuronic acid and N-acetyl glucosamine connected with 1→4 bondings). We selected native K5 (fully unsulfated), partially N-sulfated K5 (0.5 sulfate/disaccharide), O-sulfated K5 (1.5 sulfates/disaccharide), and N + O–sulfated K5 (2.5 sulfates/disaccharide). As shown in Figure 6b, both native K5 and partially N-sulfated K5 did not compete with heparin-albumin binding of PCSK9. In contrast, both O-sulfated K5 and N + O–sulfated K5 equally competed for PCSK9 binding efficiently. This shows that apparently O-sulfated K5 is an efficient PCSK9 binding molecule, which cannot further improve by additional N-sulfation. Interestingly, O-sulfated K5 is a more efficient PCSK9 binding polysaccharide compared with heparin. Furthermore, chondroitin sulfate A and C and dermatan sulfate did not compete for the binding of PCSK9 to heparin-albumin (Figure 6c). These data are in line with the data published by Gustafsen *et al.*[30] At last, to evaluate the eventual potential application of heparin-related polysaccharides as potential PCSK9 blockers, we also tested fucoidan and dextran sulfate next to heparin and O-sulfated K5 (Figure 6d). Both fucoidan and dextran sulfate strongly competed with heparin albumin for binding with PCSK9. For details on the molecular mass, degree of sulfation, and PCSK9 competition of all used polysaccharides, we refer to Supplementary Table S3. Altogether, these data demonstrate that the sulfation degree and chain length determine the binding affinity of PCSK9 to heparin.

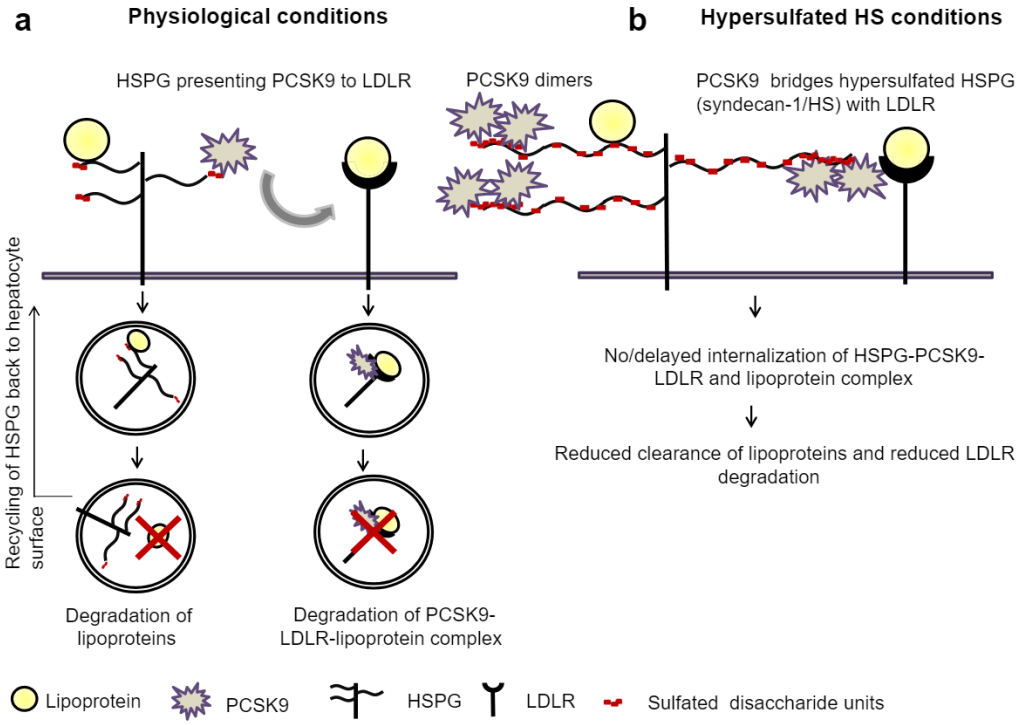


**Figure 8 | Proposed model representing potential novel mechanism for proteinuric dyslipidemia.** (**a**) Heparan sulfate (HS) proteoglycan (HSPG) presenting proprotein convertase subtilisin kexin type 9 (PCSK9) to low-density lipoprotein receptor (LDLR) in normal physiological conditions. (**b**) Formation of the HSPG-PCSK9-LDLR complex due to the hypersulfated state of HS in proteinuric conditions.

### PCSK9 colocalizes with hypersulfated HS

To investigate a potential association of PCSK9 with hypersulfated HS *in vivo*, a confocal immunofluorescence staining of PCSK9 and anti-HS mAb 3G10 was performed. As shown in Figure 7b, PCSK9 partially colocalizes to HS in the hepatic sinusoidal regions, exclusively in proteinuric animals endowed with hypersulfated HS and not in control rats lacking these hypersulfation modifications (Figure 7a).

## DISCUSSION

Although increased plasma PCSK9 was reported before in experimental[33,42] and human kidney failure,[32,43] none studied hepatic PCSK9 distribution and its relation to hepatic HS. In this study, we found that dyslipidemia in proteinuric rats is associated with hypersulfation of hepatic HS along with sinusoidal accumulation of PCSK9, without changes in LDLR protein levels.

A clearance defect in our rat model is suggested by significant increased concentrations of non-HDLc, and an unchanged cholesterol synthesis (no changes in mRNA expression of *Acat1*, *Acat2*, *Mvd*, *Hmgcr*, *Hmgcs1*, *Mvk*, and *Sqle*; Supplementary Table S1). Our data support recent studies reporting defective lipoprotein catabolism as a key cause of nephrotic dyslipidemia.[6,44–46]

In other renal disease models (puromycin aminonucleoside nephrosis rat model, genetic Imai rat model, and 5/6 nephrectomy rat model), albuminemia, hypertension, and loss of renal function are observed with proteinuria.[33,42,47–51] These rat and mouse models of the nephrotic syndrome showed reduction of hepatic LDLR at the protein level.[33,42,47–51] Loss of hepatic LDLR in these models was associated with increased PCSK9 expression.[33,42,51] Interestingly, a recent study published by Molina-Jijon *et al.* showed that PCSK9—expressed and secreted by renal cortical collecting duct epithelial cells—initiates hypercholesterolemia in nephrotic syndrome in rodents.[52,53] On the contrary, Adriamycin-treated rat model is a pure proteinuria model with no signs of edema, albuminemia, or hypertension and with unaltered serum creatinine and creatinine clearance. These animals show no other signs of nephrotic syndrome, except dyslipidemia, without changes in expression of hepatic LDLR and PCSK9 plasma levels. Based on these findings, we speculate that hypersulfation of syndecan-1/HS with sinusoidal PCSK9 accumulation, leading to hampered lipoprotein clearance and dyslipidemia, might be relatively an early event, over time followed by loss of LDLR when proteinuria/renal failure progresses into full nephrotic syndrome. Our findings strongly suggest that proteinuria is the underlying cause of dyslipidemia, HS hypersulfation, and changes in PCSK9 localization. Similar hypersulfation of HS has been reported before in cartilage and chondrosarcoma.[54,55] We previously showed hypersulfation of renal HS on various renal conditions, like ischemia/reperfusion, transplantation, and proteinuria.[27,29,56,57] Considering various mediators, growth factors, and proinflammatory cytokines related to renal conditions, we also investigated possible mediator(s) driving induction of hypersulfation in proteinuric condition. Based on

the work of Chen *et al.*, Kim *et al.*, and Suranyi *et al.*, we measured serum adiponectin and tumor necrosis factor-α levels.[58–60] Adiponectin and tumor necrosis factor-α levels can be increased in proteinuric conditions and inhibit SULF2 expression, thereby increasing HS sulfation.[58–60] However, no increment was found in both serum adiponectin and tumor necrosis factor-α levels in Adriamycin-treated rats (data not shown). Therefore, the mediator(s) inducing the hypersulfation in proteinuric conditions needs further investigation.

Hence, we propose a novel mechanism of nephrotic dyslipidemia involving PCSK9–hypersulfated HS (Figure 8). HSPGs, most likely syndecan-1, bind to PCSK9 in both proteinuric and healthy animals. But, in proteinuric conditions, the strength of PCSK9–syndecan-1/HS complex formation is increased due to highly sulfated HS. Such HSs have multiple trisulfated disaccharide units, facilitating PCSK9 dimerization along HS, because PCSK9 can form dimers in concentration-dependent manner.[61] Thus, formed dimers may bind to HS with increased affinity through multimerization avidity effect. HS binding domain in PCSK9 differs from LDLR binding domain,[30,37] allowing PCSK9 to bridge syndecan-1/HS and neighboring LDLR, forming LDLR-PCSK9-syndecan-1/HS complexes. Such complex formations were reported before between HSPG–growth factor–growth factor receptors and HSPG–chemokine–chemokine receptors.[62–64] These complexes (LDLR-PCSK9-syndecan-1/HS complexes) either get immobilized on the surface of hepatocytes or their internalization gets highly delayed, as reported before for growth factors and chemokines.[62,64–66] This makes syndecan-1 and LDLRs nonfunctional as clearance receptor by either preventing or reducing their internalization. These complexes thus might trap (V)LDLs on the surface of hepatocytes, because (V)LDL binding sites in both LDLR and free HS chains of syndecan-1 are still available. This drastically increases accumulation of triglyceride-rich remnant lipoproteins. Our model, therefore, explains elevated levels of TGs and non-HDLc in proteinuric animals without changes in syndecan-1 and LDLR. LRP-1, however, might still be functional in proteinuric condition but unable to fully compensate. We did not investigate the possible effects of proteinuria on lipoprotein composition (for instance, oxidation of LDLc).[67] Therefore, its eventual effects on receptor binding need to be investigated in future studies.

Current treatments for dyslipidemia target intestinal cholesterol uptake and endogenous cholesterol synthesis, but they are not adequate to control dyslipidemia and lower CVD-related deaths in CKD.[68] Targeting PCSK9 has emerged as a novel approach to treat hypercholesterolemia.[69–73] Other potential novel therapies are heparin mimetics. Heparin and related compounds are known to lower plasma TG concentrations by releasing lipoprotein lipase into plasma.[74,75] However, not only TGs but also TC reduce under heparin treatment, which cannot be explained by a lipoprotein lipase–releasing mechanism.[76,77] Our data suggest that, in addition to lipoprotein-lipase release, heparinoids might form a complex with plasma PCSK9, restricting PCSK9 from binding with syndecan-1/HS. Heparin and related compounds are highly cost-effective compared with current PCSK9 inhibitors. Further studies on heparin mimetics will be needed to block their pleotropic nature, without affecting their binding to PCSK9.[78,79] In this respect, we observed that nonanticoagulant RO-heparin, O-sulfated K5, N + O–sulfated K5, fucoidan, and dextran sulfate effectively compete with heparin-albumin for PCSK9 binding. Some of these polysaccharides could be administered chronically to dyslipidemic renal patients without risk of interrupting the blood clotting system. Specificity of such polysaccharides and their long-term safety need further research. Altogether, inhibition of PCSK9, either by PSCSK9 inhibitors or heparin mimetics at an early stage of nephrotic syndrome, might reduce or delay the development of and also the progression of kidney damage.[52]

In conclusion, our study provides novel insight on how hypersulfated HSPG-PCSK9 complex formation might decrease the hepatic uptake of lipoproteins, leading to dyslipidemia in proteinuric conditions. It also opens new venues for future development of heparin mimetics as PCSK9 inhibitors.

## DISCLOSURE

All the authors declared no competing interests.

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## SUPPLEMENTARY MATERIAL

Supplementary File (Word)

**Table S1.** Primer sequences and mRNA measurements by qRT-PCR of enzymes involved in HS and cholesterol synthesis in control rats and Adriamycin-treated rats.

**Table S2.** Immunofluorescence staining protocols.

**Table S3.** Characteristics of heparin(oids) and related polysaccharides with their percentage inhibition of PCSK9 binding with immobilized heparin-albumin at 20 μg/ml.

**Supplementary Methods S1.** PCSK9 competition assay.

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