Partial recovery of the endothelial glycocalyx upon rosuvastatin therapy in patients with heterozygous familial hypercholesterolemia

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Abstract The endothelial glycocalyx has been shown to serve as a protective barrier between the flowing blood and the vessel wall in experimental models. The aim of this study was to evaluate whether hypercholesterolemia is associated with glycocalyx perturbation in humans, and if so, whether statin treatment can restore this. We measured systemic glycocalyx volume (V_G) in 13 patients with heterozygous familial hypercholesterolemia (FH) after cessation of lipid-lowering therapy for a minimum of 4 weeks and 8 weeks after initiating rosuvastatin therapy. Normocholesterolemic subjects were used as controls. V_G was estimated by subtracting the intravascular distribution volume of a glycocalyx permeable tracer (dextran 40) from that of a glycocalyx impermeable tracer (labeled erythrocytes). V_G in untreated FH patients [LDL 225 ± 57 mg/dl (mean ± SD)] was significantly reduced compared with controls (LDL 93 ± 24 mg/dl) (V_G 0.8 ± 0.3 vs. 1.7 ± 0.6, respectively, $P < 0.001$). After normalization of LDL levels (95 ± 33 mg/dl) upon 8 weeks of statin treatment, V_G recovered only partially (V_G 1.1 ± 0.4 L, $P = 0.04$). The endothelial glycocalyx is profoundly reduced in FH patients, which may contribute to increased atherogenic vulnerability. This perturbation is partially restored upon short-term statin therapy.—Meuwese, M. C., H. L. Mooij, M. Nieuwdorp, B. van Lith, R. Marck, H. Vink, J. J. P. Kastelein, and E. S. G. Stroes. **Partial recovery of the endothelial glycocalyx upon rosuvastatin therapy in patients with heterozygous familial hypercholesterolemia.** ***J. Lipid Res.*** **2009.** 50: **148–153.**

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Endothelial cells are shielded from direct exposure to the flowing blood by a highly hydrated mesh of macromolecules, named the endothelial glycocalyx (1). Its major components include proteoglycans with their associated glycosaminoglycans, such as hyaluronan and heparan sulfate, as well as glycoproteins bearing acidic oligosaccharides with terminal sialic acids. Recent intravital microscopic studies showed that the endothelial glycocalyx is 0.5 to 3 μm thick (2, 3). Several decades ago, Gorog and Born (4) already found that sialic acid density in rabbits was decreased in predilection sites for atherosclerosis. These findings have now been corroborated, because loss of glycocalyx leads to a wide spectrum of vascular abnormalities in experimental models. These comprise increased vascular permeability as well as increased adhesion of leukocytes and thrombocytes to the vessel wall (5–8). Restoration of the glycocalyx is associated with reversal of these proatherogenic changes (5). Collectively, there is growing evidence that the endothelial glycocalyx plays a central role in vascular homeostasis and could be of importance in protecting the vasculature against atherogenic insults.

Recently, our group developed a novel technique to estimate the volume of the endothelial glycocalyx in humans. Using this method, Nieuwdorp et al. (9) showed that acute hyperglycemia results in a profound perturbation of the glycocalyx, coinciding with vascular dysfunction and activation of the coagulation system. Glycocalyx loss was also shown to be present in patients with type 1 diabetes mellitus. Damage was most severe in patients with microalbuminuria (10, 11). In experimental models, other risk factors such as oxygen radical stress, inflammation, and exposure to oxidized low-density lipoprotein (oxLDL) have also been shown to disrupt the glycocalyx (8, 12–14).

In the present study we evaluated whether hypercholesterolemia is associated with glycocalyx perturbation in

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Abbreviations: CRP, C-reactive protein; FH, familial hypercholesterolemia; Ht, hematocrit; OPS, orthogonal polarization spectroscopy; oxLDL, oxidized low-density lipoproteins; V_G, systemic glycocalyx volume.

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humans, and if so, whether statin treatment is able to reverse these derangements. For this purpose we selected patients with heterozygous familial hypercholesterolemia (FH), characterized by elevated LDL-cholesterol levels.

METHODS

Study population

We enrolled 13 nonsmoking male patients with heterozygous FH (DNA diagnosis and/or strong clinical suspicion based on lipid profile; family history; and/or presence of xanthomas, xanthelasmata or corneal arcus) without a history of cardiovascular disease or diabetes mellitus. Thirteen normocholesterolemic, nonsmoking, healthy male subjects served as a control group. All subjects gave written informed consent, and approval was obtained from the internal review board of the Academic Medical Center. The study was carried out in accordance with the principles of the Declaration of Helsinki.

Study design

We measured systemic glycocalyx volume (V_G), safety and lipid profiles, and glycocalyx related parameters in FH patients after cessation of statin therapy for a minimum period of 4 weeks and 8 weeks after initiating intensive statin treatment (rosuvastatin 40 mg, QD). In view of the exploratory nature of the present experiment and the increased risk of cardiovascular disease in FH patients, a placebo-controlled trial was deemed unethical at this stage. Normocholesterolemic subjects were used as controls. All experiments were performed after an overnight fast. Participants were asked to refrain from heavy physical exercise 24 h prior to the study visit. Blood pressure was measured three times, from which the mean of the last two measurements was used as systolic and diastolic blood pressure. All data and images were analyzed by lab staff and readers unaware of the clinical details and or stage of the protocol.

Estimation of endothelial glycocalyx volume

The endothelial glycocalyx allows limited access to plasma macromolecules and erythrocytes, whereas smaller tracers can permeate into the glycocalyx (15). We estimated V_G by subtracting circulating plasma volume from the intravascular distribution volume of a glycocalyx permeable tracer (i.e., neutral dextran 40), as previously published (9, 10, 16). The intravascular distribution volume of labeled autologous erythrocytes was used to quantify circulating blood volume (17). In our hands, this method has an intersession coefficient of variation of 16 ± 12% (18).

In summary, two cannulas were inserted in the antecubital veins of both forearms for the collection of blood and infusion of dextran 40 as well as labeled autologous erythrocytes. To quantify circulating plasma volume, 50 ml blood was drawn and centrifuged. Subsequently, 250 mg/ml of sodium fluorescein was added to the erythrocyte fraction for 5 min. After washing, labeled erythrocytes were resuspended in saline to the initial volume and reinfused. Blood samples were drawn before infusion as well as 4, 5, 6, and 7 min after infusion. The circulating fraction of labeled erythrocytes was measured using flowcytometry (FACSCalibur; Becton Dickinson, Mountain View, CA) to estimate the total circulating erythrocyte volume (V_{ERY}). Circulating plasma volume was calculated from V_{ERY} and large vessel hematocrit (Ht) by the following formula: $([1-Ht] \times V_{ERY})/Ht$.

Dextran 40 was used as a probe to estimate the intravascular volume including the glycocalyx compartment. A bolus of 10 ml dextran 1 (Promiten; NPBI International, Emmercompascuum, the Netherlands) was injected to attenuate the risk of anaphylactic reactions. Subsequently, 100 ml dextran 40 kDa (Rheomacrodex; NPBI International, Emmercompascuum, the Netherlands) was injected intravenously, followed by repeated blood sampling at 3, 5, 7, 10, 15, 20, and 30 min. Dextran 40 concentration was calculated by measuring the increase in glucose concentration in the post infusion samples after hydrolyzation of dextran 40 glucose polymers, correcting for background glucose levels. Glucose concentration per time point was assessed in duplicate using the hexokinase method. To determine the initial intravascular distribution volume of dextran 40, the concentration of dextran 40 at the time of injection was estimated by exponential fitting of the measured dextran 40 concentrations. Exponential time constants (τ [min]) were used to determine dextran 40 systemic clearance rates (τ-1 [min-1]).

Thickness of endothelial glycocalyx in individual capillary blood vessels was measured by orthogonal polarization spectral (OPS) imaging of the sublingual microcirculation (Cytometrics, Philadelphia, PA) (18). Briefly, the width of flowing erythrocytes was measured in five individual capillaries before and immediately after leukocyte passage. In healthy capillaries, the glycocalyx limits capillary blood filling by separating erythrocytes from the luminal endothelial surface. Since leukocytes transiently compress the capillary endothelial glycocalyx, the corresponding transient widening of the capillary erythrocyte column can be used to estimate capillary glycocalyx dimension (19). Analysis of the images was performed with ImageJ (National Institutes of Health, USA) by a single observer, unaware of the clinical details of the participants. In our hands, this method has an intersession coefficient of variation of 15 ± 5% (18).

Biochemical parameters

Total cholesterol, HDL-cholesterol, and triglycerides were measured by standard enzymatic methods (Roche Diagnostics, Basel, Switzerland). LDL-cholesterol was calculated using the Friedewald formula. OxLDL was measured using a commercially available ELISA (Mercodia, Uppsala, Sweden). Alanine aminotransferase and aspartate aminotransferase were measured by pyridoxalphosphate activation assay (Roche Diagnostics). Creatinin was measured by Jaffé kinetic colorimetric test (Roche Diagnostics) on Modular P800 (Roche Diagnostics). Glucose was assessed using the hexokinase method (Gluco-quant, Hitachi 917; Hitachi). HbA1C was measured by HPLC (Reagens Bio-Rad Laboratories, Veenendaal, the Netherlands) on a Variant II (Bio-Rad Laboratories). Plasma C-reactive protein (CRP) levels were measured with a commercially available assay (Roche, Switzerland). Ht was measured after centrifugation of heparinized blood at 10,000 rpm for 5 min (Hettich, Tuttlingen, Germany). For further analysis, plasma aliquots were snap-frozen and stored at −80°C. Quantitative total plasma hyaluronan levels were measured by ELISA (Echelon Biosciences, Salt Lake City, UT) as was syndecan-1 (Diaclone, Besançon, France). Plasma hyaluronidase activity was determined with a previously described assay (20).

Statistical analysis

Results are expressed as means ± SD. Differences between normocholesterolemic and hypercholesterolemic subjects were tested using an unpaired Student's *t*-test (two-tailed). Differences within the hypercholesterolemic group with and without treatment were tested using a paired Student's *t*-test (two-tailed). CRP and triglyceride levels are generally not normally distributed. Therefore, we present medians (interquartile range) and used nonparametric tests for these values. The relation between V_G as dependent variable and other parameters was explored using

TABLE 1. Clinical characteristics of hypercholesterolemic and normocholesterolemic subjects

	FH patients	FH patients	Controls		
	No treatment (n = 13)	Rosuvastatin (n = 13)	No treatment (n = 13)	P[a]	P[b]
Age (yrs)	38.5 ± 9.2		33.2 ± 13.5	ns	
BMI (kg/m^2)	24.4 ± 1.6	24.4 ± 1.6	22.9 ± 1.8	ns	ns
Systolic blood pressure (mmHg)	134 ± 12	127 ± 11	124 ± 10	0.04	0.02
Diastolic blood pressure (mmHg)	82 ± 10	80 ± 8	67 ± 9	<0.001	ns
Total cholesterol (mg/dl)	288 ± 56	159 ± 35	166 ± 26	<0.001	<0.001
LDL-cholesterol (mg/dl)	225 ± 57	95 ± 33	93 ± 24	<0.001	<0.001
HDL-cholesterol (mg/dl)	45 ± 12	47 ± 18	58 ± 11	0.005	ns
Triglycerides (mg/dl)	89 (76–120)	68 (63–90)	34 (33–50)	0.001	ns
OxLDL (U/L)	122 ± 37	61 ± 17	80 ± 17	<0.001	<0.001
Glucose (mmol/L)	4.9 ± 0.3	4.8 ± 0.5	5.1 ± 0.4	ns	ns
CRP (mg/L)	0.7 (0.5–1.6)	1.0 (0.4–5.6)	0.3 (0.3–0.8)	0.03	ns
Leukocyte count (×10^9/L)	5.3 ± 1.4	5.2 ± 1.2	5.3 ± 0.9	ns	ns

BMI, body mass index; oxLDL, oxidized LDL. Values are presented as means ± SD. Triglycerides and C-reactive protein (CRP) are presented as median (interquartile range) and tested nonparametrically, as they are generally not normally distributed.

[a] P value familial hypercholesterolemia (FH) patients (no treatment) vs. controls (unpaired Student's *t*-test).

[b] P value FH patients (no treatment) vs. FH patients (rosuvastatin treatment) (paired Student's *t*-test).

Pearson's or Spearman's correlation coefficient. Analyses were performed with SPSS version 11.5 (Chicago, IL). A P value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics

Clinical characteristics of the participants are listed in **Table 1**. As expected, FH patients who had discontinued statin treatment for a duration of 4 weeks had substantially higher LDL-cholesterol levels than the normocholesterolemic controls (LDL-cholesterol mean ± SD: 225 ± 57 mg/dl vs. 93 ± 24 mg/dl, respectively). HDL-cholesterol levels, triglyceride levels, blood pressure, as well as CRP were all within the normal range, but significantly less favorable in FH patients compared with controls. Age, body mass index (BMI), as well as plasma glucose levels were comparable between groups.

Glycocalyx volume and hypercholesterolemia

V_G in untreated FH patients was substantially lower than in the normocholesterolemic control group (0.8 ± 0.3 lsignificance vs. 1.7 ± 0.6 lrespectively, $P < 0.001$) (**Fig. 1**).

In normocholesteromic controls V_G was correlated with plasma hyaluronan levels ($r = 0.577$, $P = 0.039$). In FH patients without treatment V_G negatively correlated with leukocyte count ($r = -0.601$, $P = 0.03$), plasma hyaluronidase activity ($r = -0.588$, $P = 0.035$), as well as systolic blood pressure ($r = -0.680$, $P = 0.011$). Within the groups, there were no significant correlations between V_G and age, BMI, HDL-cholesterol, LDL-cholesterol, oxLDL, triglycerides, plasma glucose, HbA1c, or CRP.

Glycocalyx volume after statin treatment

After 8 weeks treatment with rosuvastatin 40 mg QD LDL-cholesterol levels completely normalized to 95 ± 33 mg/dl (Table 1). V_G recovered partially (V_G 1.1 ± 0.4 L, $P = 0.04$). Capillary endothelial glycocalyx thickness,

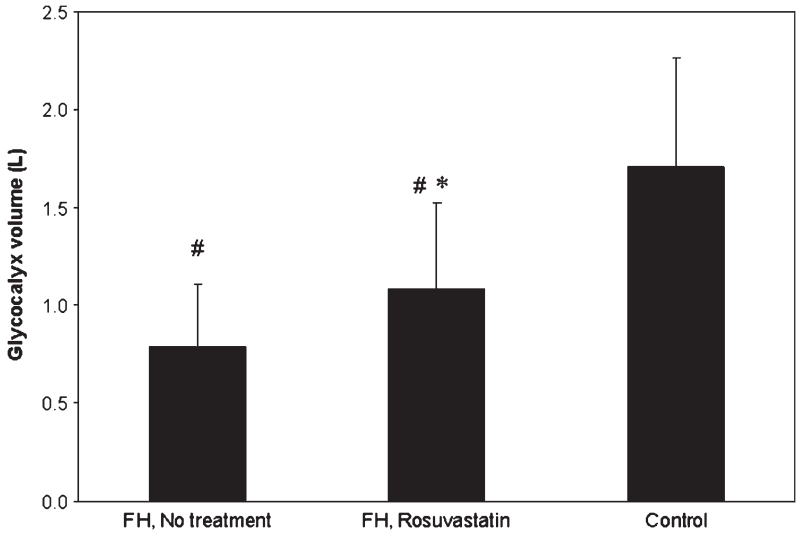

Fig. 1. Systemic glycocalyx volume (V_G) in hypercholesterolemic and normocholesterolemic subjects. V_G is significantly reduced in FH patients compared with normocholesterolemic controls. Statin therapy partially restored glycocalyx volume. Mean ± SD.

determined using OPS imaging, followed a similar pattern. Mean glycocalyx thickness was 0.4 ± 0.1 μm in patients without treatment and increased to 0.5 ± 0.1 μm after treatment ($P = 0.17$). Unfortunately, OPS images could only be analyzed in 8 out of 13 patients and 9 out of 13 controls due to technical difficulties. After treatment, there was no statistically significant inverse correlation between V_G and leukocyte count ($r = -0.523$, $P = 0.067$). Besides that, the inverse correlation between change in V_G and change in leukocyte count did not reach statistical significance ($\rho = -0.492$, $P = 0.087$). There was no significant correlation between (change in) V_G and LDL-cholesterol. In normocholesterolemic controls, mean glycocalyx thickness was 0.5 ± 0.2 μm.

Vascular permeability and glycocalyx-associated parameters

The clearance rate of dextran 40 is an indirect indicator of vascular permeability. The steepest slope of the clearance curves was found in FH patients without treatment (−0.014), compared with −0.006 in normocholesterolemic controls ($P = 0.07$) (**Fig. 2**). Rosuvastatin treatment did not significantly alter the dextran clearance rate (slope −0.012). Hyaluronan, the main component of the glycocalyx, was significantly higher in FH patients on treatment compared with controls ($P < 0.001$) (FH, no treatment 73.5 ± 30.1 ng/ml; FH, rosuvastatin 80.0 ± 17.1; controls, no treatment 60.1 ± 10.6). A similar pattern was observed in plasma hyaluronidase activity (FH, no treatment 264 ± 197 U; FH, rosuvastatin 655 ± 252; Controls, no treatment 234 ± 135; FH, no treatment or control vs. FH, rosuvastatin, $P < 0.001$). There were no significant differences in plasma syndecan-1 levels.

DISCUSSION

In the present study we found a substantial reduction of V_G in patients with FH compared with normocholesterolemic controls. After 8 weeks of intensive statin treatment, LDL-cholesterol levels completely normalized, yet V_G only partially recovered. V_G in FH patients was correlated with leukocyte count. Taking into account the cumulating evidence that glycocalyx perturbation enhances vulnerability of the vessel wall, our findings imply that novel strategies aimed at restoring V_G may be of interest to further optimize vascular resistance toward atherogenic insults.

Concomitant with the decreased glycocalyx volume, dextran 40 clearance was increased in FH patients, although not reaching statistical significance. The endothelial glycocalyx has been shown to be a central orchestrator of capillary permeability by serving as a macromolecular barrier covering the intercellular junctions. For example, removal of sialic acids, a major component within the glycocalyx, led to increased uptake of LDL in the vessel wall (24). Increased vascular permeability has previously been described in FH patients (25). Glycocalyx damage could contribute to this and thus to premature atherosclerosis, which is often seen in these patients.

FH is known to be associated with increased oxidant stress (21, 22), illustrated by increased oxLDL levels in our patients, as well as increased inflammatory mediators, such as CRP (26). Both oxLDL as well as inflammatory mediators have been shown to have a detrimental impact on glycocalyx in experimental models (8, 14, 23). In fact, increased oxygen radical stress has been suggested to be one of the principal mediators of glycocalyx perturbation. This is illustrated by the findings that glycocalyx damage upon oxLDL exposure in hamsters could be prevented by radical scavengers (8). Similarly, we previously found that glycocalyx damage by hyperglycemia in humans could be restored by infusion of the antioxidant N-acetylcysteine (10). Unfortunately, statin therapy resulted in partly restoration of the endothelial glycocalyx only.

Although LDL and oxLDL levels normalized after rosuvastatin treatment, V_G only partially recovered. A possible explanation is the short duration of treatment (8 wk) in light of the lifelong exposure to high cholesterol levels in FH patients. In contrast, many studies have shown rapid recovery of endothelial function upon statin treatment (27, 28). Moreover, (change in) glycocalyx volume did not

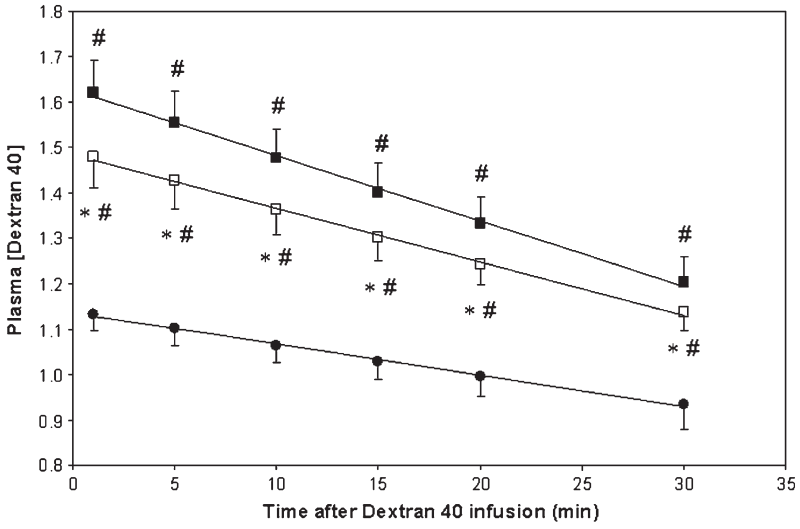

Fig. 2. Dextran clearance in hypercholesterolemic and normocholesterolemic subjects. The dextran clearance, an indicator of vascular permeability, was fastest in FH patients without treatment, although not statistically significant. The slope was −0.014 in FH patients, without treatment, −0.012 in FH patients on rosuvastatin, and −0.006 in normocholesterolemic controls (FH patients, no treatment vs. controls, $P = 0.07$). Mean ± SEM. Black squares: FH patients, no treatment; white squares: FH patients, rosuvastatin; black circles: normocholesterolemic controls, no treatment.

significantly correlate with LDL-cholesterol levels. Correlate with leukocyte count failed to reach statistical significance. This may imply a role of the inflammatory activity, rather than LDL-cholesterol in glycocalyx perturbation. On the other hand, it has recently been suggested that endothelial "memory" exists, as it was shown that vascular stress, mediated by oxidants, persisted following glucose normalization after a hyperglycemic period (29). This may also be applicable to hypercholesterolemia-induced vascular stress. It could imply that hypercholesterolemia has longer lasting effects on the endothelium as well as the endothelial glycocalyx. Short-term lowering of cholesterol may not be sufficient to overcome this endothelial memory. Further studies are needed to evaluate whether long-term statin therapy is able to establish further restoration of the endothelial glycocalyx.

Obviously, statins have not been designed to restore the endothelial glycocalyx. Notably, we even observed an increase in hyaluronidase levels after rosuvastatin therapy. As hyaluronidase breaks down a major component of the glycocalyx (i.e., hyaluronan) increased levels could attenuate restoration despite the effective lowering of LDL-cholesterol. Whereas the exact cause of statin-associated increase in hyaluronidase is unclear, statins are known to upregulate KLF2, which in turn has been shown to increase hyaluronidase expression (30, 31). If restoration of the glycocalyx is to be reached, more direct targeting is likely to be achieved by modulating glycosaminoglycan metabolism, either by supporting glycosaminoglycan production or by preventing degradation. Such compounds showing larger effects on the endothelial glycocalyx may offer further protection toward atherogenic insults on top of statins.

Study limitations

This study has a small sample size. Therefore, we chose to include a homogeneous group of healthy, nonsmoking, male FH patients and normocholesterolemic control subjects with an overt difference in LDL-cholesterol. Whereas other baseline clinical characteristics also showed minor differences, these were all well within normal range. Second, the role of the endothelial glycocalyx as a target of damage and, thus, as a structure deserving protection, is just starting to emerge. Although the techniques to evaluate the endothelial glycocalyx in humans are new, indirect, and under constant development, we believe that our data provide valuable information to build initial hypotheses. The lack of a difference between local and systemic glycocalyx estimations can reflect either a power problem in this limited series of observations or relate to the fact that local, sublingual glycocalyx thickness may, to some extent, be affected independently from V_G. Thus, van den Berg et al. (23) showed large differences in glycocalyx thickness between different locations in the vasculature within one animal in wild-type as well as hypercholesterolemic mice. Most importantly, improvements following statin use could be observed using either method. The accuracy of glycocalyx volume estimates is largely determined by the accuracy of dextran 40 distribution volume estimates. Because of its small size and neutral charge, dextran 40 is also cleared from the circulation. Therefore, we estimated the intravascular dextran 40 concentrations before vascular leakage or renal clearance by extrapolating dextran 40 concentrations to the time of injection. Third, we did not treat normocholesterolemic subjects with rosuvastatin 40 mg. Therefore, we are unable to evaluate the effect of statin on the glycocalyx in absence of hypercholesterolemia.

CONCLUSION

The endothelial glycocalyx is profoundly reduced in FH patients, which may contribute to increased atherogenic vulnerability. This perturbation is partially restored upon short-term statin therapy. Awaiting trials validating the protective role of an intact glycocalyx, the present findings may suggest a need for novel interventions aimed at additional restoration of the glycocalyx.

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