Targeting the vessel wall in cardiovascular prevention
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Chapter 4

Effect of sulodexide on endothelial glycocalyx and albumin permeability in type 2 diabetic patients

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Work in progress, original research
ABSTRACT

Background: Vascular complications are the major cause of morbidity and mortality in patients with type 2 diabetes (DM2). Loss of the endothelial glycocalyx, a protective layer covering the vessel wall, has been suggested to contribute to increased vascular permeability as well as the increased propensity to vascular complications in DM2. In the present study, we set out to evaluate (i) whether DM2 is associated with loss of endothelial glycocalyx and if so, (ii) whether supplementation of glycocalyx constituents (with sulodexide, a mixture of heparan sulphate/dermatan sulphate) can restore glycocalyx volume and vascular barrier function.

Methods: We determined glycocalyx volume, transcapillary escape rate of albumin (TERalb) and urine albumin content in 21 male DM2 patients and 14 normoglycemic controls. Measurements were repeated in diabetics after 8 weeks treatment with 200 mg sulodexide.

Results: Glycocalyx volume in DM2 patients tended to be reduced compared to controls (9.7 ± 5.2 vs. 14.0 ± 5.8 mL/kg in DM2 and controls respectively; p = 0.04). This was accompanied by higher TERalb values in DM2 than controls (5.1 ± 2.3% vs. 3.5 ± 1.7 %, respectively; p = 0.05). After 8 weeks of sulodexide, glycocalyx volume in DM2 patients was 11.3 ± 6.2 mL/kg (ns vs. controls), whereas TERalb decreased (4.6 ± 3.0%; ns vs. controls). Sulodexide did not significantly alter urine albumin excretion.

Conclusion: DM2 was associated with a loss of glycocalyx volume and increased vascular permeability compared to controls. Sulodexide partially normalized glycocalyx volume and TERalb. The present findings imply that restoration of the glycocalyx may be a promising target to restore the increase in vascular permeability associated with hyperglycemia.

Keywords: sulodexide, endothelial glycocalyx, diabetes mellitus type 2, microalbuminuria, vascular permeability

Abbreviations: DM1, diabetes mellitus type 1; DM2, diabetes mellitus type 2; TERalb, transcapillary escape rate of albumin
INTRODUCTION

Diabetes mellitus is characterized by an increased propensity to vascular complications. Microvascular complications such as retinopathy and nephropathy, as well as macrovascular complications, such as coronary artery disease, are largely responsible for morbidity and mortality in type 2 diabetic (DM2) patients (1). An early sign of vascular damage is increased vascular permeability as well as increased renal leakage of albumin, i.e. microalbuminuria. According to the Steno-hypothesis, albuminuria reflects widespread vascular damage (2). Indeed, microalbuminuria is associated with an almost twofold increased risk of cardiovascular disease (3). Although the underlying mechanisms responsible for microalbuminuria as well as its link with cardiovascular complications are multicausal, hyperglycemia is likely to be a causal factor (4).

We recently showed that acute hyperglycemia results in a profound perturbation of the endothelial glycocalyx, coinciding with vascular dysfunction and activation of the coagulation system (5). Moreover, we found that patients with type 1 diabetes mellitus (DM1) are characterized by a reduction of glycocalyx volume of almost 50%. Damage was most severe in patients with concomitant microalbuminuria (6).

The endothelial glycocalyx is a 0.5 to 3 μm thick layer, comprising proteoglycans with their associated glycosaminoglycans such as hyaluronan, heparan sulphate and dermatan sulphate (7, 8). It shields the endothelium from direct exposure to the flowing blood (9). Loss of glycocalyx leads to a wide spectrum of vascular abnormalities in experimental models. These include increased vascular permeability as well as increased adhesion of leukocytes and thrombocytes to the vessel wall (10-13). Restoration of the glycocalyx is associated with reversal of these pro-atherogenic changes (10). These findings have led to the hypothesis that reversal of glycocalyx damage may provide an attractive therapeutic target to prevent vascular complications.

However, to date no drugs are available with the capacity to specifically improve glycocalyx volume and/or function. In vitro studies have suggested that supplementation of glycocalyx constituents may have the capacity to restore damage (10, 11). In this respect, sulodexide is an interesting compound. Sulodexide consist of a mixture of 80% heparan sulphate and 20% dermatan sulphate, both constituents of the glycocalyx. In fact, we recently showed that sulodexide, a mixture of 80% heparan sulphate and 20% dermatan sulphate, was able to attenuate hyperglycemia-associated endothelial permeability for albumin in vitro (14). This was accompanied by partial restoration of the glycocalyx layer. In parallel, preliminary trials have shown that sulodexide decreases urinary albumin leakage a in diabetic patients, the mechanism of which remains to be elucidated (15-17).
Based on these results, we hypothesized that oral administration of sulodexide could reduce vascular permeability in DM2 patients, in part due to restoration of the glycocalyx layer. Therefore, we set out to evaluate (i) whether DM2 is associated with loss of endothelial glycocalyx and if so, (ii) whether supplementation with sulodexide can restore and vascular barrier function, as assessed by the transcapillary escape rate of albumin (TERalb).

**METHODS**

**Study population**

We enrolled 21 non-smoking, male patients with diabetes mellitus type 2 without overt signs of macrovascular disease (defined as a history of myocardial infarction, stroke, peripheral vascular disease or signs of macrovascular disease at physical examination). Fourteen normoglycemic, non-smoking, 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 glycocalyx volume, transcapillary escape rate (TER) of albumin and urinary albumin excretion in all participants. In diabetic patients, all measurements were repeated after 8 weeks supplementation with sulodexide (KRX-101, 200 mg). Sulodexide is a natural extract from bowel mucosa, which contains a mixture of 80% low-molecular mass heparan sulphate and 20% dermatan sulphate. All experiments were performed after an overnight fast. Participants were asked to refrain from heavy physical exercise 24 h prior to the study visit. Statin therapy was discontinued temporarily. Blood pressure was measured three times, from which the mean of the last two measurements was used as systolic and diastolic blood pressure.

**Estimation of endothelial glycocalyx volume**

The endothelial glycocalyx allows limited access to plasma macromolecules and erythrocytes, whereas smaller tracers can permeate into the glycocalyx (18, 19). We estimated systemic endothelial glycocalyx volume by subtracting circulating plasma volume from the intravascular distribution volume of a glycocalyx permeable tracer, i.e. neutral dextran 40, as previously published (5, 6). The intravascular distribution volume of labeled, autologous erythrocytes was used to quantify circulating blood volume (20). 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 minutes. After washing, labeled erythrocytes were resuspended in saline to the initial volume and re-infused. Blood samples were drawn before infusion as well as 4, 5, 6, and 7 minutes 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 (\(V_{\text{ery}}\)). Circulating plasma volume was calculated from \(V_{\text{ery}}\) and large vessel hematocrit (Ht) by the following formula: \((1 \text{– Ht}) \times V_{\text{ery}})/\text{Ht}\).

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 minutes. 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 (\(\tau\) [min]) were used to determine dextran 40 systemic clearance rates (\(\tau^{-1}\) [min\(^{-1}\)]). This method has an intersession coefficient of variation of 16% (21).

**Transcapillary Escape Rate of albumin**

Microvascular permeability was determined by the transcapillary escape rate of \(^{125}\text{-\textit{I}}\)–albumin (TER\textit{alb}). \(^{125}\text{-\textit{I}}\)–labeled albumin solution of 100 kBq in 5 ml saline was infused as an intravenous bolus. Blood samples were drawn from the contralateral arm at baseline, and at 10, 15, 20, 30, 45 and 60 minutes. Plasma radioactivity was measured in each sample and a urine sample using a scintillation detector (automatic γ-counter). TER-alb was expressed as the percentage decline in plasma radioactivity from 10 to 60 minutes after injection.

**Biochemical parameters**

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). Albumin content in urine was determined after 24 hours collection. Plasma C-reactive protein (CRP) levels were measured with a commercially available assay (Roche, Switzerland). Hematocrit (Ht) was measured after centrifugation of heparinized blood at 10,000 rpm for 5 minutes (Hettich, Tuttingen, Germany). Total cholesterol, HDL-cholesterol, and triglycerides were measured by standard enzymatic methods (Roche Diagnostics, Basel, Switzerland). LDL-cholesterol was calculated using the Friedewald formula. Alanine aminotransferase and aspartate aminotransferase were measured by
pyridoxalphosphate activation assay (Roche Diagnostics). Creatinin was measured by Jaffe’ kinetic colorimetric test (Roche Diagnostics) on Modular P800 (Roche Diagnostics). For further analysis, plasma aliquots were snap-frozen and stored at -80°C.

Statistical analysis
Results are expressed as means ± SD. Differences between normoglycemic and diabetic subjects were tested using an unpaired Student’s t test (two-tailed). Differences within the diabetic group with and without treatment were tested using a paired Student’s t test (two-tailed). Urine albumin levels, CRP and triglyceride levels are generally not normally distributed. Therefore, we present medians (interquartile range) and used non parametric tests for these values. The relation between glycocalyx volume and TERalb and other parameters was explored using Spearman’s correlation coefficient. Analyses were performed with SPSS version 11.5 (Chicago, IL, USA). A p value < 0.05 was considered statistically significant.

RESULTS

Clinical characteristics
Clinical characteristics of the participants are listed in Table 1. Eight out of 21 DM2 patients presented with increased urinary albumin excretion (> 30 mg/day). The median level of albumin in all diabetics was 11 [0 - 72] mg/24 hr, with 0 [0 - 11] mg/d in normoalbuminuric patients and 118 [46 - 353] mg/d in patients with increased urinary albumin, compared to 0 [0 - 10] mg/d in controls (p = 0.09). Treatment of DM2 patients with sulodexide had no significant effect on albuminuria. After treatment, the median urine albumin excretion was 16 [0 - 60] mg/d (p = 0.5 compared to baseline), with 0 [0 - 15] mg/d in normoalbuminuric patients (p = 1.0 compared to baseline) and 85 [60 - 245] mg/24 hr in those with increased urinary albumin.

Figure 1. Endothelial glycocalyx volume in type 2 diabetic patients and normoglycemic subjects
Effect of sulodexide in diabetes mellitus type 2

Furthermore, no significant changes in HbA1c, glucose levels, PT or aPTT were observed upon sulodexide treatment.

Table 1. Clinical characteristics of type 2 diabetic patients and normoglycemic subjects

<table>
<thead>
<tr>
<th></th>
<th>DM2 patients Baseline</th>
<th>DM2 patients 8 wks sulodexide</th>
<th>Controls Baseline</th>
<th>Controls 8 wks sulodexide</th>
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<tbody>
<tr>
<td></td>
<td>n=21</td>
<td>n=19</td>
<td>n=14</td>
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<tr>
<td>Age, yrs</td>
<td>54.7 ± 6.2</td>
<td>54.7 ± 6.2</td>
<td>57.2 ± 7.2</td>
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<td></td>
<td>p = 0.30</td>
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<tr>
<td>BMI, kg/m²</td>
<td>29.2 ± 4.4</td>
<td>29.2 ± 4.4</td>
<td>26.9 ± 2.2</td>
<td>26.9 ± 2.2</td>
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<tr>
<td></td>
<td>p = 0.09</td>
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<tr>
<td>Systolic blood pressure, mmHg</td>
<td>147 ± 19</td>
<td>144 ± 26</td>
<td>134 ± 18</td>
<td>134 ± 18</td>
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<tr>
<td></td>
<td>p = 0.05</td>
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<tr>
<td>Diastolic blood pressure, mmHg</td>
<td>89 ± 9</td>
<td>84 ± 13</td>
<td>81 ± 8</td>
<td>81 ± 8</td>
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<td></td>
<td>p = 0.04</td>
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<td>Glucose, mmol/L</td>
<td>7.6 ± 3.3</td>
<td>7.4 ± 2.4</td>
<td>4.9 ± 0.7</td>
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<td></td>
<td>p = 0.05</td>
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<tr>
<td>HbA1c, %</td>
<td>6.7 ± 1.7</td>
<td>7.0 ± 1.1</td>
<td>5.5 ± 0.3</td>
<td>5.5 ± 0.3</td>
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<td>p = 0.01</td>
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<tr>
<td>Urine albumin excretion, mg/d</td>
<td>11 [0-72]</td>
<td>16 [0-60]</td>
<td>0 [0-10]</td>
<td>0 [0-10]</td>
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<td></td>
<td>p = 0.09</td>
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<td>Total cholesterol, mmol/L</td>
<td>5.4 ± 1.1</td>
<td>5.2 ± 1.2</td>
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<td></td>
<td>p = 0.41</td>
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<td>LDL-cholesterol, mmol/L</td>
<td>3.4 ± 0.9</td>
<td>3.2 ± 1.0</td>
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<td></td>
<td>p = 0.14</td>
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<tr>
<td>HDL-cholesterol, mmol/L</td>
<td>1.0 ± 0.3</td>
<td>1.0 ± 0.3</td>
<td>1.3 ± 0.3 &lt;0.01</td>
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</tr>
<tr>
<td>Triglycerides, mmol/L</td>
<td>1.7 [1.1-3.1]</td>
<td>1.8 [0.9-3.2]</td>
<td>1.0 [0.9-1.4]</td>
<td>1.0 [0.9-1.4]</td>
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<tr>
<td>CRP, mg/L</td>
<td>2.0 [0.6-3.9]</td>
<td>1.3 [0.5-3.2]</td>
<td>1.1 [0.5-1.4]</td>
<td>1.1 [0.5-1.4]</td>
</tr>
</tbody>
</table>

Values are presented as means ± SD. Urine albumin excretion, triglycerides and CRP are presented as median [interquartile range] and tested non-parametrically, as they are generally not normally distributed.

P*: Baseline values of DM2 patients vs. values after 8 weeks sulodexide (paired Student’s t test or Wilcoxon, when appropriate)

P**: Baseline values of DM2 patients vs. controls (unpaired Student’s t test or Mann Whitney, when appropriate)

(p = 0.4 compared to baseline). Furthermore, no significant changes in HbA1c, glucose levels, PT or aPTT were observed upon sulodexide treatment.

Endothelial glycocalyx volume at baseline

Plasma volume was comparable between diabetic patients and normoglycemic controls (DM2: 2.7 ± 0.7 L vs. controls 2.7 ± 0.5 ml, p = 0.9), as was plasma hematocrit (0.42 ± 0.03 vs. 0.42 ± 0.03, respectively, p = 0.9). The distribution volume of dextran 40 kDa was 3.6 ± 0.6 L in diabetics compared to 3.8 ± 0.8 L in controls (p = 0.4). This resulted in an average glycocalyx volume of 0.9 ± 0.5 L in DM2 patients and 1.2 ± 0.5 L in controls (p = 0.05). Normalized per kg bodyweight, we observed a glycocalyx volume 9.7 ± 5.2 mL/kg in DM2 patients, compared to 14.0 ± 5.8 mL/kg in controls (p = 0.04). This difference was more pronounced in diabetics with (micro)albuminuria (7.8 ± 2.4 mL/kg, p = 0.02 compared to controls), than in diabetics without microalbuminuria (10.8 ± 6.1 mg/kg, p = 0.2) (Figure 1).
Endothelial glycocalyx volume upon sulodexide administration

After 8 weeks treatment with sulodexide, average plasma volume tended to increase in diabetic patients to \(2.9 \pm 0.8 \text{ L}(p = 0.07\text{ compared to baseline})\). This was accompanied by a decrease in hematocrit from \(0.42 \pm 0.03\) to \(0.40 \pm 0.02\ (p = 0.002)\). Dextran distribution volume tended to increase to \(3.8 \pm 1.1 \text{ L}(p = 0.07)\). This resulted in an average glycocalyx volume of \(1.0 \pm 0.5 \text{ L}(p = 0.2\text{ compared to controls})\). Normalized per kg bodyweight, we observed values comparable to our controls in diabetics without microalbuminuria \((14.5 \pm 4.8 \text{ mL/kg}, p = 0.9\text{ compared to controls}),\) but low values in diabetics with microalbuminuria \((4.9 \pm 2.8, p = 0.002\text{ compared to control})\) (Figure 1). Overall, mean glycocalyx volume in diabetics was \(11.3 \pm 6.2 \text{ mL/kg}(p = 0.2)\).

Transcapillary Escape Rate of Albumin

The transcapillary escape rate of albumin (TERalb) was \(5.1 \pm 2.3\%\) in the first hour in DM2 patients vs. \(3.5 \pm 1.7\%\) in controls \((p = 0.05)\). After treatment with sulodexide, this difference was no longer significant (TERalb \(4.6 \pm 3.0\%, p = 0.3\text{ compared to controls})\) (Figure 2). Glycocalyx volume in controls was inversely correlated with TERalb \((\rho = -0.60, p = 0.05)\). Following sulodexide, glycocalyx volume was negatively correlated with urine albumin excretion \((p = -0.47, p = 0.05)\). TERalb did not correlate with urine albumin content in diabetic patients \((p = -0.148, p = 0.52)\) or controls \((p = -0.016, p = 0.96)\). It was inversely correlate with HbA1c in DM2 patients after sulodexide treatment \((p = -0.61, p = 0.005)\).

DISCUSSION

In patients with DM2 endothelial glycocalyx volume was decreased compared to controls with a concomitant increase in transcapillary escape rate of albumin. Following 8 weeks of
sulodexide, glycocalyx volume in diabetic patients was no longer different from non-diabetic controls. Markedly, improvement was absent in patients with (micro)albuminuria. Furthermore, sulodexide reduced the transcapillary escape rate of albumin. However, this was not accompanied by a reduction of urinary albumin excretion. The present findings imply that restoration of glycocalyx in DM2 patients may lead to a decreased vascular permeability in DM2. Further studies are warranted to evaluate whether glycocalyx restoration also has the capacity to reduce the propensity towards vascular complications in diabetic patients.

Glycocalyx perturbation in DM2
In the present study, we corroborate our previous findings of loss of glycocalyx volume in patients with DM1, particularly in those with microalbuminuria (5). We observed similar trends in DM2. Interestingly, the difference in glycocalyx volume in DM2 patients appeared to be less pronounced compared to patients with DM1. This could be due to the fact that DM1 patients have endured longer exposure to hyperglycemia. Alternatively, it should be taken into account that DM2 patients as well as their controls were older and more obese compared to the previously reported DM1 patients and controls. We previously reported that BMI is inversely related to glycocalyx volume, which could result in less marked differences between groups (19).

Glycocalyx and sulodexide
Duling and coworkers have previously shown that supplementation of constituents of the glycocalyx, particularly hyaluronan, has the ability to protect the vessel wall from insults by restoring the protective capacities of the endothelial glycocalyx (10, 22). Recently, we were able to show that supplementation of endothelial cells with sulodexide in vitro reversed the increased transendothelial albumin leakage under hyperglycemic conditions. Markedly, barrier restoration was accompanied by increased glucosamine staining of the cultured endothelial cells, indicating recovery of the endothelial glycocalyx layer (14). Our present findings confirm these results. However, sulodexide did not improve glycocalyx volume and vascular permeability in all patients. Especially, in patients with microalbuminuria restoration of systemic glycocalyx volume was absent. These findings imply that, perhaps, at a certain level of damage, restoration with sulodexide may prove to be unrealistic. In contrast, at earlier stages, prevention is promising.

Glycocalyx and vascular permeability
Mehta and Malik provided experimental evidence to show that the glycocalyx is instrumental in determining the endothelial barrier function under physiological conditions (23). The abundant presence of negatively charged glycosaminoglycans, such as heparan sulphate, in the endothelial glycocalyx has been shown to contribute profoundly to the charge-selective repulsion of negatively charged proteins, such as albumin. In fact, alteration in either the pro-
duction or sulphation degree of heparan sulphate is thought to contribute to the increased permeability of both the kidney (24-26) as well as the systemic vascular barrier in diabetic patients (2). The latter is supported by the increased vascular leakage of albumin that we observed in patients compared to controls.

**Urine albumin excretion and sulodexide**

After a relatively short period of treatment of 8 weeks of a small number of patients, we did not observe a reduction of urinary albumin excretion. Although our study can be criticized for its limited duration and size, recently two clinical trials were terminated due to disappointing results following six to twelve months of sulodexide administration (27). Both in DM2 patients with persistent microalbuminuria as well as patients with overt diabetic nephropathy sulodexide failed to show significant reduction in urinary protein excretion. However, it should be kept in mind that systemic transcapillary escape rate in diabetes may respond differently towards drug intervention than urinary albumin excretion as they reflect different pathogenetic mechanisms (28). Changes in urinary albumin excretion have been suggested to reflect predominantly hemodynamic changes and thus glomerular capillary pressure, rather than true restoration of structural damage to the glomeruli and/or the glomerular basement membrane (29). Therefore, lack of reversal of (micro)albuminuria does not exclude an impact on systemic permeability of the vessel wall. Moreover a possible effect of sulodexide may not be confined to microalbuminuric patients alone. Finally it must be kept in mind that urinary albumin excretion naturally fluctuates as much as 40% (29).

**Study limitations**

This study has several limitations. First, we cannot exclude that the 200 mg dose of sulodexide was suboptimal. We chose this dose based on dose-response curves in vitro (14). Improvements in the endothelial barrier already occurred at relatively low concentration of sulodexide of 0.06 μg/mL, which is lower than the plasma concentrations following oral dosing of 200 mg (30). Moreover, further dose escalation will, at some point, be limited by the anticoagulant effects of heparan sulphates, resulting in aPTT prolongation (30). Second, sulodexide only contains two constituents of the glycocalyx, i.e. heparan sulphate and dermatan sulphate. Other constituents, such as hyaluronan, have been shown to be pivotal in restoring glycocalyx properties (10, 22). In addition, there are indications that shedding of hyaluronan is increased in diabetic patients (31). Therefore, future studies need to address whether different mixtures of glycocalyx constituents have a larger impact on glycocalyx perturbation.

**Conclusion**

DM2 is associated with a decrease in endothelial glycocalyx volume and increased vascular permeability, which may contribute to vascular complications. Sulodexide moderately improved glycocalyx volume and vascular leakage of albumin, but not urine albumin excretion.
The present findings imply that restoration of the glycocalyx may be a promising target to restore the increase in vascular permeability associated with hyperglycemia. This calls for the search of novel therapeutics aimed at glycocalyx barrier function to improve vascular protection.

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