

Effect of sulodexide on endothelial glycocalyx and vascular permeability in patients with type 2 diabetes mellitus

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Abstract

Aims/hypothesis Endothelial glycocalyx perturbation contributes to increased vascular permeability. In the present study we set out to evaluate whether: (1) glycocalyx is perturbed in individuals with type 2 diabetes mellitus, and (2) oral glycocalyx precursor treatment improves glycocalyx properties.

Methods Male participants with type 2 diabetes ($n=10$) and controls ($n=10$) were evaluated before and after 2 months of sulodexide administration (200 mg/day). The glycocalyx dimension was estimated in two different vascular beds using sidestream dark field imaging and combined fluorescein/indocyanine green angiography for sublingual and

retinal vessels, respectively. Transcapillary escape rate of albumin (TER_{alb}) and hyaluronan catabolism were assessed as measures of vascular permeability.

Results Both sublingual dimensions (0.64 [0.57 – 0.75] μm vs 0.78 [0.71 – 0.85] μm , $p<0.05$, medians [interquartile range]) and retinal glycocalyx dimensions (5.38 [4.88 – 6.59] μm vs 8.89 [4.74 – 11.84] μm , $p<0.05$) were reduced in the type 2 diabetes group compared with the controls whereas TER_{alb} was increased ($5.6\pm 2.3\%$ vs $3.7\pm 1.7\%$ in the controls, $p<0.05$). In line with these findings, markers of hyaluronan catabolism were increased with diabetes (hyaluronan 137 ± 29 vs 81 ± 8 ng/ml and hyaluronidase 78 ± 4 vs 67 ± 2 U/ml, both $p<0.05$). Sulodexide increased both the sublingual and retinal glycocalyx dimensions in participants with diabetes (to 0.93 [0.83 – 0.99] μm and to 5.88 [5.33 – 6.26] μm , respectively, $p<0.05$). In line, a trend towards TER_{alb} normalisation (to $4.0\pm 2.3\%$) and decreases in plasma hyaluronidase (to 72 ± 2 U/ml, $p<0.05$) were observed in the diabetes group.

Conclusion/interpretation Type 2 diabetes is associated with glycocalyx perturbation and increased vascular permeability, which are partially restored following sulodexide administration. Further studies are warranted to determine whether long-term treatment with sulodexide has a beneficial effect on cardiovascular risk.

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Keywords Diabetes mellitus type 2 · Endothelial glycocalyx · Hyaluronan · Sulodexide · Vascular permeability

Abbreviations

ARB	Angiotensin-receptor blocker
CRP	C-reactive protein
FAG	Fluorescein angiography
ICG	Indocyanine green angiography
GAG	Glycosaminoglycan
GlcNAc	<i>N</i> -acetyl-glucosamine
SDF	Sidestream dark field
TER _{alb}	Transcapillary escape rate of albumin

Introduction

Diabetes mellitus is characterised by an increased propensity towards vascular complications. Microvascular complications, such as retinopathy and nephropathy, as well as macrovascular complications are largely responsible for morbidity and mortality in type 2 diabetes mellitus patients [1–3]. An early sign of vascular damage is increased vascular permeability, which may eventually be associated with microalbuminuria at a later stage. Although the pathophysiology leading to increased vascular permeability and microalbuminuria and the link with cardiovascular complications have not been fully elucidated, hyperglycaemia is likely to be a causal factor [4].

The endothelial glycocalyx layer consisting of proteoglycans with their associated glycosaminoglycans (GAGs) [5–9], covers the luminal side of each vessel wall. Under physiological conditions, the glycocalyx shields the endothelial lining from direct contact with circulating blood elements [3]. Recently, we observed that acute hyperglycaemia resulted in a profound perturbation of the endothelial glycocalyx, which coincided with vascular dysfunction and activation of the coagulation system in healthy volunteers [5]. In fact, with respect to chronic hyperglycaemia, patients with type 1 diabetes mellitus were characterised by a 50% decrease in glycocalyx volume. Loss of glycocalyx volume closely correlated with increased plasma hyaluronidase levels, indicative of enhanced hyaluronan catabolism [10]. Glycocalyx perturbation has been associated with a wide spectrum of vascular abnormalities in experimental models, including increased vascular permeability as well as increased adhesion of leucocytes and thrombocytes [11, 12]. In animal models, restoration of the glycocalyx reversed these abnormalities [11]. This led to the concept that reversal of glycocalyx damage may be an attractive therapeutic target capable of preserving vascular integrity. In vitro studies have suggested that supplementation of glycocalyx constituents in part restored glycocalyx damage through both increased *N*-acetyl-glucosamine (GlcNAc)-driven GAG synthesis and decreased GAG catabolism [13, 14]. Sulodexide is a

commercially available compound consisting of heparan sulphate (80%) and dermatan sulphate (20%). Sulodexide is degraded in the digestive tract into GlcNAc building blocks, leading to an increase in the precursors available for GAG synthesis [14, 15]. In vitro studies have shown that sulodexide attenuates hyperglycaemia-associated endothelial permeability in vitro through enhanced GAG synthesis [16], but the effect of sulodexide treatment on diabetes-associated microalbuminuria in vivo is less clear. In the present study, we thus hypothesised that increasing the precursors available for GAG synthesis could improve endothelial glycocalyx properties in type 2 diabetes mellitus patients. To this end, we evaluated the glycocalyx dimension in two different vascular beds before and after oral administration of sulodexide for 8 weeks.

Methods

Study population In this investigator-initiated study, we enrolled ten non-smoking men with diabetes mellitus type 2 without microalbuminuria (based on 24 h urine microalbuminuria measurement in the 6 months preceding the study), retinopathy or macrovascular disease (defined as a history of myocardial infarction, stroke, peripheral vascular disease or signs of macrovascular disease at physical examination). All patients used oral antihyperglycaemic medication and patients using antihypertensive medication were excluded from the study. Statins were discontinued at least 4 weeks prior to study initiation. Ten normoglycaemic non-smoking age-matched healthy men served as an age-matched control group. Participants were asked to refrain from heavy physical exercise 24 h prior to the study visit. Alcohol, caffeine and metformin were withheld at least 12 h before the study. All participants gave written informed consent, and approval was obtained from the internal review board of the Academic Medical Centre. The study was registered in the Netherlands Trial Register (NTR780/ISRCTN82695186). The study was carried out in accordance with the principles of the Declaration of Helsinki.

Study design In patients and age-matched controls we measured: (1) local sublingual glycocalyx thickness using sidestream dark field (SDF) imaging; (2) retinal glycocalyx thickness using fluorescein and indocyanine green angiography (FAG/ICG); (3) transcapillary escape rate of albumin (TER_{alb}); and (4) circulating plasma levels of hyaluronan and its degrading enzyme hyaluronidase both at baseline and after 8 weeks of sulodexide administration (200 mg/day; 25 mg/capsule, Alfa Wasserman, Milan, Italy). Sulodexide is a glycosaminoglycan of natural origin extracted from mammalian intestinal mucosa, containing a mixture of 80% low-molecular-mass heparan sulphate and

20% dermatan sulphate [15]. Blood pressure was measured three times, from which the means of the last two measurements were used as systolic and diastolic blood pressure values.

Endothelial glycocalyx dimension We assessed the endothelial glycocalyx dimension of both the sublingual and the retinal circulations. The determination of the erythrocyte–endothelium gap is the gold standard for glycocalyx measurement in vivo [17] as the endothelial glycocalyx allows limited access to erythrocytes. Using this principle, the sublingual glycocalyx dimension was estimated using SDF imaging [17]. Briefly, in each individual approximately 1,000 measurement sites of 10 μm in length were marked in sublingual vessels. At each measurement site, multiple estimates of the erythrocyte column width were made by measuring both the median erythrocyte width as well as the 90th percentile of erythrocyte width distribution. For vessels with diameters ranging from 10 to 20 μm , a functional estimate of glycocalyx dimension was estimated by comparing the 50th percentile of erythrocyte width with the 90th percentile of erythrocyte width (Fig. 1). Based on these estimates, a single median value for glycocalyx dimension was calculated for each individual. The reproducibility of SDF measurements used to estimate glycocalyx dimension is good within our centre, with an intersession coefficient of variation of $5.6 \pm 3.2\%$ ($n=10$ controls measured on two separate occasions). The same principle can be applied to retinal endothelial glycocalyx measurement with fluorescence angiography. This method uses the intravascular distribution of two different fluorescent tracers: fluorescein and indocyanine green [18]. Comparable with the exclusion of erythrocytes, the endothelial glycocalyx also allows limited access to plasma macromolecules, whereas smaller tracers can readily permeate into the glycocalyx [19]. In the retinal capillaries, fluorescein fills up the entire vascular compartment while indocyanine green binds to albumin and is therefore excluded by the glycocalyx layer. By calculating the difference between the two compartments, the dimension of the glycocalyx layer in retinal vessels can be estimated. Retinal glycocalyx dimensions, represented by the indocyanine green exclusion zone on retinal angiography, were determined in larger retinal vessels (diameter on FAG: 90 μm and above), thereby avoiding the heterogeneity of glycocalyx dimensions in smaller vessels.

Transcapillary escape rate of albumin

Vascular permeability was determined by the transcapillary escape rate of ^{125}I -labelled albumin (TER_{alb}) [20]. ^{125}I -

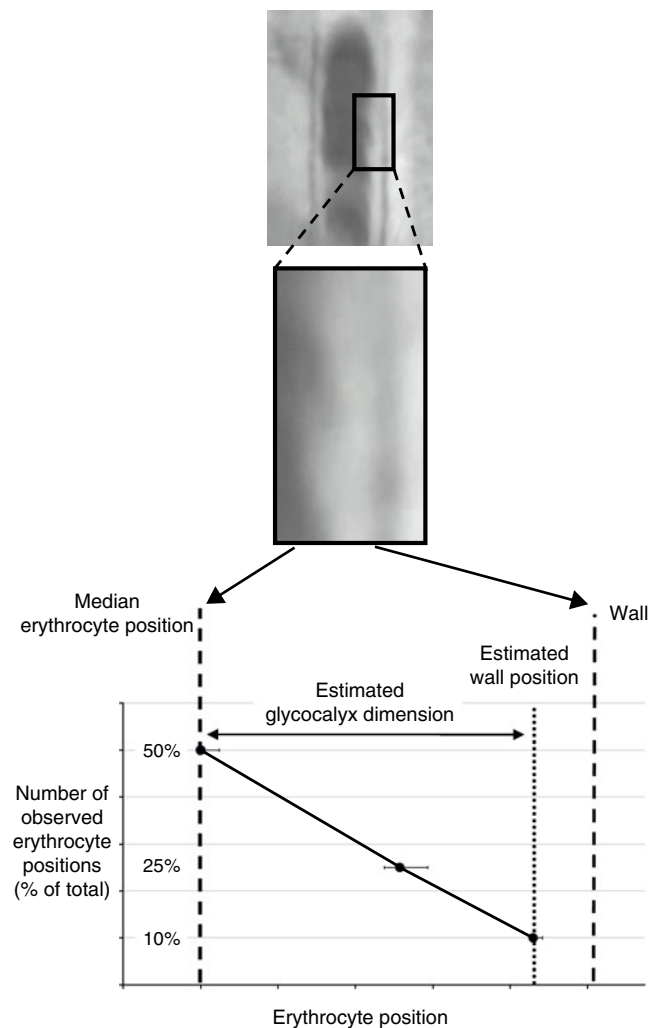


Fig. 1 Measurement of sublingual glycocalyx dimension using SDF imaging

labelled 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 5, 10, 15, 20, 30, 45 and 60 min. Plasma radioactivity was measured in each sample and in a urine sample, collected at the end of the procedure, using a scintillation detector (automatic γ -counter). TER_{alb} was expressed as the percentage decline in plasma radioactivity from 10 to 60 min after injection.

Biochemical variables

Glucose was assessed using the hexokinase method (Glucoquant, Hitachi 917; Hitachi, Valbonne, France). $\text{HbA}_{1\text{c}}$ was measured by HPLC (Reagens Bio-Rad Laboratories, Veenendaal, the Netherlands) on a Variant II system (Bio-Rad Laboratories, Hemel Hempstead, UK). Plasma C-reactive protein (CRP) levels were measured with a commercially available assay (Roche Diagnostics, Basel, Switzerland).

Total cholesterol, HDL-C, and triacylglycerols were measured by standard enzymatic methods (Roche Diagnostics). LDL-C was calculated using the Friedewald formula. Alanine aminotransferase and aspartate aminotransferase were measured by pyridoxalphosphate activation assay (Roche Diagnostics). Creatinine 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 . Quantitative total plasma hyaluronan levels were measured by enzyme-linked immunosorbent assay (Echelon Biosciences, Salt Lake City, UT, USA) [21]. Plasma hyaluronidase levels were determined with an assay as described previously by Nieuwdorp et al. [10].

Statistical analysis

Results are expressed as means \pm SD. Baseline differences between controls and patients with type 2 diabetes were tested using an unpaired Mann–Whitney test (two-tailed). Differences within groups before and after treatment were tested using a paired Student's *t* test (two-tailed). CRP and triacylglycerols were not normally distributed. Therefore, we present medians (interquartile ranges) and used non-parametric tests for these values. Analyses were performed

with SPSS version 11.5 (Chicago, IL, USA). A *p* value < 0.05 was defined as statistically significant.

Results

Clinical characteristics Clinical characteristics of the participants are listed in Table 1. As compared with controls, patients with type 2 diabetes were characterised by increased glycaemic indices, diastolic blood pressure as well as increased CRP levels. No significant changes in BMI, HbA_{1c} or glucose levels were observed with sulodexide administration in patients and controls. Other than an increased incidence of epistaxis in one healthy volunteer, no side effects were reported.

Endothelial glycocalyx thickness measured with SDF imaging and FAG/ICG Patients with type 2 diabetes mellitus were characterised by reduced sublingual glycocalyx dimensions compared with the controls at baseline: 0.64 (0.57 – 0.75) μm (type 2 diabetes) vs 0.78 (0.71 – 0.85) μm (controls; $p < 0.05$ type 2 diabetes vs controls; Fig. 2). Following sulodexide treatment the average sublingual glycocalyx thickness increased in diabetic patients (to 0.93 [0.83 – 0.99] μm ; $p < 0.05$ placebo vs sulodexide; Fig. 3). In controls, sulodexide had no significant effect

Table 1 Baseline characteristics of participants

Characteristic	Participants with type 2 diabetes			Controls			
	Baseline <i>n</i> =10	After 8 weeks of sulodexide <i>n</i> =10	<i>p</i> ^a	Baseline <i>n</i> =10	<i>p</i> ^b	After 8 weeks of sulodexide <i>n</i> =10	<i>p</i> ^c
Age (years)	51.9 \pm 8.4	51.9 \pm 8.4	<i>p</i> =NS	56.3 \pm 8.5	<i>p</i> =NS	56.3 \pm 8.5	<i>p</i> =NS
Duration of diabetes (years)	5.1 \pm 5.6	5.1 \pm 5.6	<i>p</i> =NS				
BMI (kg/m ²)	29.7 \pm 5.5	29.6 \pm 5.3	<i>p</i> =NS	27 \pm 2.1	<i>p</i> =NS	26.9 \pm 1.8	<i>p</i> =NS
SBP (mmHg)	141 \pm 14	139 \pm 18	<i>p</i> =NS	135 \pm 15	<i>p</i> =NS	141 \pm 27	<i>p</i> =NS
DBP (mmHg)	87 \pm 8	84 \pm 9	<i>p</i> =NS	82 \pm 8	<i>p</i> <0.05	81 \pm 6	<i>p</i> =NS
Glucose (mmol/l)	7.1 \pm 2.0	7.3 \pm 2.4	<i>p</i> =NS	4.9 \pm 0.4	<i>p</i> =0.002	5.2 \pm 0.6	<i>p</i> =NS
HbA _{1c} (%)	7.1 \pm 1.2	7.1 \pm 1.2	<i>p</i> =NS	5.6 \pm 0.3	<i>p</i> <0.001	5.5 \pm 0.2	<i>p</i> =NS
TC (mmol/l)	5.6 \pm 1.0	5.3 \pm 1.4	<i>p</i> =NS	6.3 \pm 1.3	<i>p</i> =NS	5.5 \pm 0.9	<i>p</i> =NS
LDL-C (mmol/l)	3.4 \pm 0.8	3.1 \pm 1.3	<i>p</i> =NS	4.4 \pm 1.1	<i>p</i> =NS	3.5 \pm 1.0	<i>p</i> =NS
HDL-C (mmol/l)	1.1 \pm 0.3	1.0 \pm 0.3	<i>p</i> =NS	1.4 \pm 0.1	<i>p</i> =0.001	1.4 \pm 0.3	<i>p</i> =NS
TG (mmol/l)	2.0 (1.4–3.5)	2.1 (1.1–3.0)	<i>p</i> =NS	1.0 (0.9–1.3)	<i>p</i> =0.001	1.3 (0.8–1.4)	<i>p</i> =NS
CRP (mg/l)	1.9 (0.5–5.5)	1.5 (0.5–4.4)	<i>p</i> =NS	1.2 (0.5–1.4)	<i>p</i> <0.05	2.6 (0.8–14)	<i>p</i> =NS

Values expressed as means \pm SD

Triacylglycerol and CRP values are presented as median (interquartile range) due to skewed distribution

DBP, diastolic blood pressure; HR, heart rate; SBP, systolic blood pressure; TC, total cholesterol; TG, triacylglycerols

^aDiabetes patients at baseline vs diabetes patients after 8 weeks of sulodexide

^bBaseline diabetes patients vs baseline controls

^cBaseline controls vs control participants after 8 weeks of sulodexide

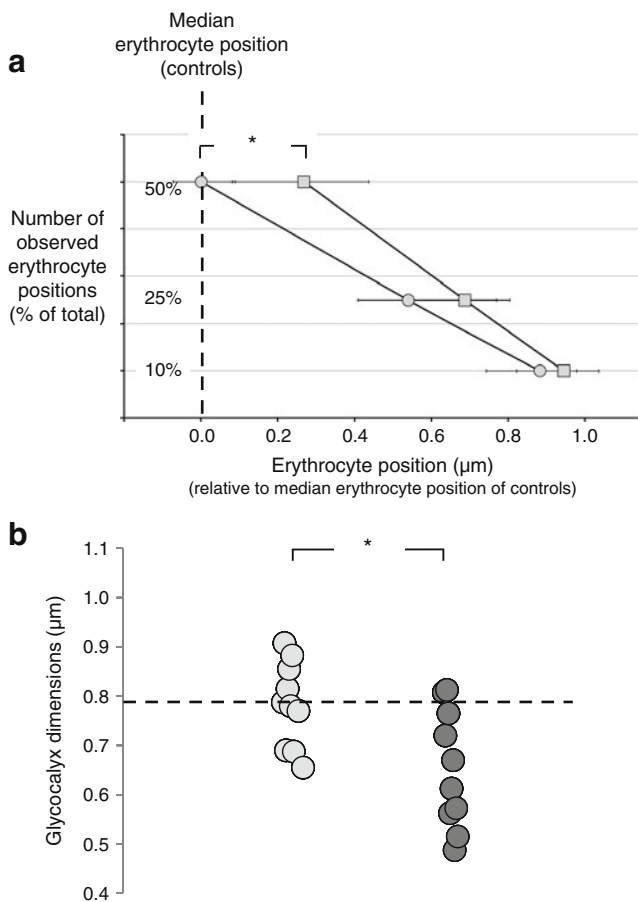


Fig. 2 Endothelial glycocalyx in control and type 2 diabetic participants. **a** Compared with controls, median erythrocyte width was significantly increased in participants with type 2 diabetes, resulting in a significant movement of erythrocytes towards the vessel wall; $*p < 0.05$. Circles, controls; squares, type 2 diabetes group. **b** Glycocalyx dimensions were reduced in the group with type 2 diabetes because of a significant increase in the median erythrocyte width, while both control and diabetic participants had similar values of the 90th percentiles of the erythrocyte column width (identifying the position of the vessel wall), $*p < 0.05$. Light circles, controls; dark circles, type 2 diabetes group

on glycocalyx dimension (increasing to $0.88 [0.80–0.99]$ μm). In a subset of patients and controls ($n=6$ per group), retinal glycocalyx dimensions were also determined both before and after 8 weeks of sulodexide administration. The retinal glycocalyx dimension was reduced in type 2 diabetes mellitus ($5.38 \mu\text{m} [4.88–6.59]$ vs controls $8.89 \mu\text{m} [4.74–11.84]$, $p < 0.05$ type 2 diabetes vs controls; Fig. 4). Following administration of sulodexide for 8 weeks, retinal glycocalyx thickness increased in participants with type 2 diabetes mellitus ($5.88 \mu\text{m} [5.33–6.26]$; $p=0.05$ vs baseline). In contrast to the effect on sublingual capillary glycocalyx, sulodexide decreased retinal glycocalyx dimension in controls (to $4.87 [3.89–6.33]$ μm , $p < 0.05$), particularly in those with a relatively large glycocalyx dimension at baseline (data not shown).

Transcapillary escape rate of albumin TER_{alb} in the first hour after infusion was increased in type 2 diabetes mellitus patients ($5.6 \pm 2.3\%$ in patients vs $3.7 \pm 1.7\%$ in controls, $p < 0.05$; Fig. 5). Following administration of sulodexide for 2 months, a trend towards normalisation of TER_{alb} was observed in those with type 2 diabetes mellitus (to $4.0 \pm 2.3\%$, $p=0.08$) whereas in controls TER_{alb} was unaffected (to $3.3 \pm 1.6\%$).

Biochemistry Plasma hyaluronan levels were higher in type 2 diabetes mellitus patients compared with healthy controls at baseline (137 ± 29 vs 81 ± 8 ng/ml, $p < 0.05$; Fig. 6). Plasma hyaluronidase levels (indicative of both endogenous hyaluronan- and glycocalyx-degrading capacity) were also increased in patients compared with controls (78 ± 4 vs 67 ± 2 U/ml, $p < 0.05$), which is in line with data from type 1 diabetes patients [10, 21]. Following sulodexide administration in participants with type 2 diabetes mellitus, plasma hyaluronidase activity decreased (from 78 ± 4 to 72 ± 2 U/l, $p < 0.05$) whereas hyaluronan levels were comparable (137 ± 29 vs 129 ± 15 ng/ml; NS). In the controls, however, hyaluronan increased (from 81 ± 8 to 107 ± 13 ng/ml, $p < 0.05$) as did plasma hyaluronidase activity (from 67 ± 2 to 71 ± 3 U/ml, $p < 0.05$) following sulodexide administration.

Discussion

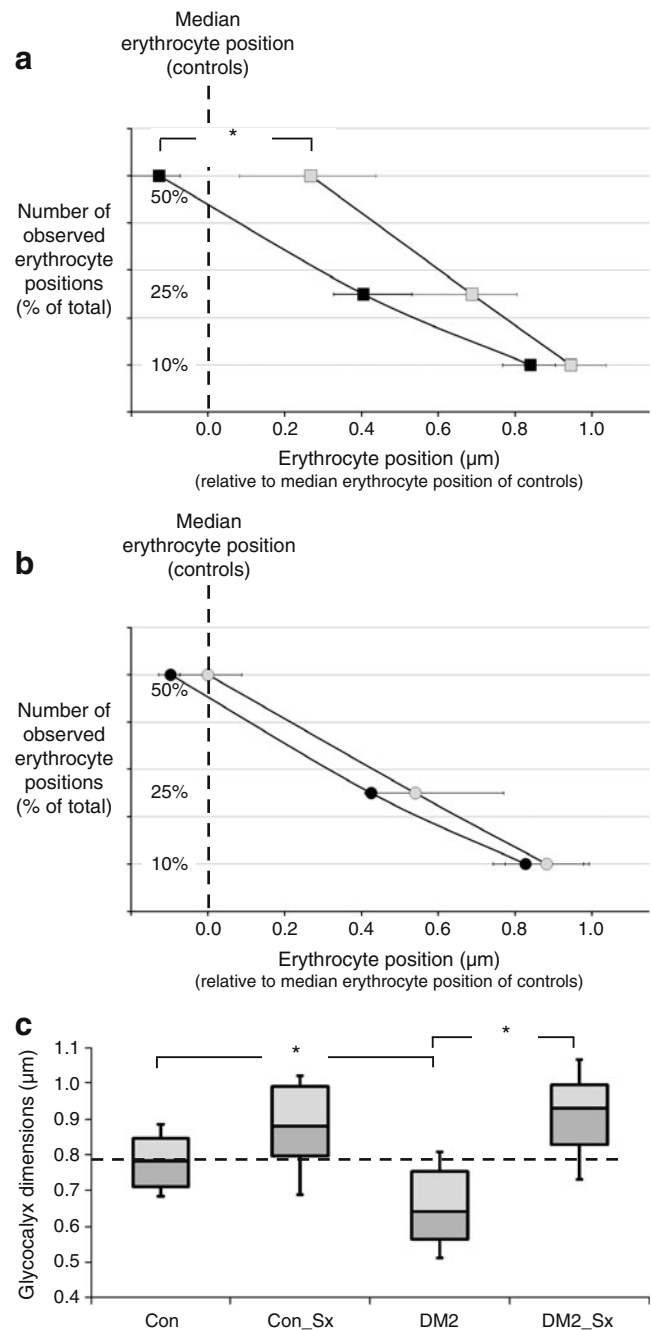
In the present study, we show that the endothelial glycocalyx dimensions of both the sublingual and the retinal microcirculation are decreased in patients with type 2 diabetes mellitus compared with healthy controls. Following 8 weeks of sulodexide administration to establish enhanced precursor abundance for GAG synthesis, glycocalyx dimensions improved in both the sublingual and the retinal microvasculature. This improvement coincided with a trend towards improvement of transcapillary albumin escape rate and a reduction in hyaluronan catabolism. These novel findings warrant further studies to evaluate whether endothelial glycocalyx restoration may be of value as an early marker predicting a risk reduction in vascular complication rate in type 2 diabetes mellitus.

Following earlier reports in individuals with type 1 diabetes mellitus, we now demonstrate the presence of glycocalyx perturbation in two different microvascular beds in patients with type 2 diabetes mellitus [10]. The significant reduction in glycocalyx dimensions in both sublingual as well as retinal vessels in type 2 diabetes underpins the systemic nature of glycocalyx perturbation. The latter coincided with a significant increase in the transcapillary escape rate of albumin indicative of increased systemic permeability [21–25], lending further support to

Fig. 3 The effect of sulodexide on endothelial glycocalyx in control and diabetic participants. **a** Following sulodexide treatment, median erythrocyte width decreased significantly in the type 2 diabetes group, resulting in a significant restoration of the distance of erythrocytes from the vessel wall; $*p < 0.05$. White squares, diabetes group before sulodexide exposure; black squares, diabetes group following sulodexide treatment. **b** Following sulodexide treatment, median erythrocyte width was not significantly changed in controls, resulting in similar distances of erythrocytes from the vessel. White circles, control group before sulodexide exposure; black circles, diabetes group following sulodexide treatment. **c** Glycocalyx dimensions at baseline and after 8 weeks of treatment with sulodexide in controls ($n=10$; con and Con_Sx, respectively) and diabetic participants ($n=10$; DM2 and DM2_Sx, respectively) measured by SDF imaging show sublingual glycocalyx dimensions were significantly reduced in type 2 diabetes, and sulodexide treatment restored microvascular glycocalyx dimensions to control values. Individual capillary glycocalyx dimensions were estimated from the transient widening of the erythrocyte column. Data are presented as whisker plots with median (interquartile range). $*p < 0.05$

the concept of systemic glycocalyx perturbation in diabetes mellitus. In this respect, experimental studies have implied that the endothelial glycocalyx may be instrumental in maintaining the endothelial barrier function under physiological conditions [11]. Alterations in the proteoglycan/glycosaminoglycan matrix structure of the endothelial glycocalyx could adversely affect the charge-selective repulsion of negatively charged proteins such as albumin, resulting in increased transvascular leakage in both kidney [26, 27] and the systemic vascular barrier in diabetes mellitus [10]. However, systemic albumin clearance (TER_{alb}) may not be the best method to evaluate changes in systemic vascular permeability, particularly in diabetes mellitus, as the impact of impaired reabsorption of albumin in the proximal tubules in diabetes mellitus may outweigh potential changes in systemic albumin leakage, as suggested by Russo et al. [28].

Oral sulodexide supplementation was associated with a significant improvement of both sublingual and retinal endothelial glycocalyx dimensions as well as a trend towards normalisation of the TER_{alb}. Mechanistically, the increased plasma hyaluronan levels and hyaluronidase levels in type 2 diabetes at baseline may reflect increased GAG catabolism in chronic hyperglycaemia [10], whereas sulodexide reduced GAG metabolism as attested to by a reduction of plasma hyaluronidase activity. Restoration of GAG metabolism has been suggested to improve the barrier function of the vasculature. Thus, Duling et al. previously showed that enhanced abundance of precursors for endothelial GAGs (particularly hyaluronan and unfractionated heparin) led to restoration of the vasculo-protective capacity of the endothelial glycocalyx [12, 29]. In the present study, oral administration of sulodexide is expected to result in enhanced precursor abundance, which may drive the correction of the endothelial glycocalyx. As low-molecular-



weight heparins may also exert beneficial effects on microalbuminuria and retinopathy in diabetes mellitus [30, 31], supplementation of other GAGs delivering comparable molar equivalents of glucosamine, galactosamine and glucuronic acid may share the beneficial properties of sulodexide for endothelial glycocalyx.

The apparent conflicting results regarding the effect of sulodexide on microalbuminuria deserve further discussion. The trials reporting a beneficial effect of sulodexide administration compared with placebo were all performed in renin-angiotensin-inhibitor-naive normotensive type 2

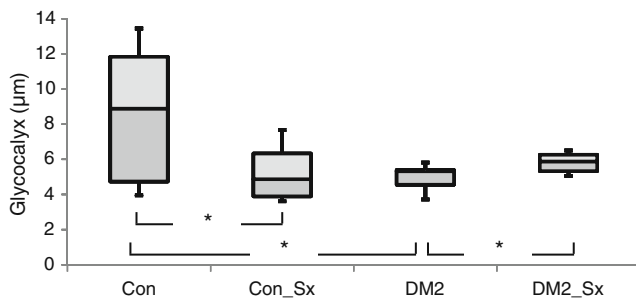


Fig. 4 The effect of sulodexide on retinal glycoalyx in control and diabetic participants. Glycoalyx dimensions in controls at baseline and after 8 weeks of treatment with sulodexide ($n=6$; con and Con_Sx, respectively) and diabetic participants ($n=6$; DM2 and DM2_Sx, respectively) measured by FAG/ICG. Individual glycoalyx dimensions were estimated by subtracting the intravascular distribution of two different fluorescent tracers: indocyanine green from fluorescein. Data are presented as whisker plots with median (interquartile range). $*p<0.05$

diabetes mellitus patients without overt vascular complications [32–34]. Recently, a beneficial effect of sulodexide was also reported that was additive to those of angiotensin-converting-enzyme inhibitors and/or angiotensin-receptor blockers (ARBs) [35]. Notably, type 2 diabetes mellitus patients in this trial were relatively young (mean age 55 years) without hypertension and with preserved renal function, and hence had less prolonged hyperglycaemic exposure. In contrast, two sulodexide trials performed in type 2 diabetes mellitus patients aiming to show protective effects of sulodexide additional to those of ARBs [36], were terminated prematurely because of lack of an effect on urinary albumin excretion [37]. However, patients included

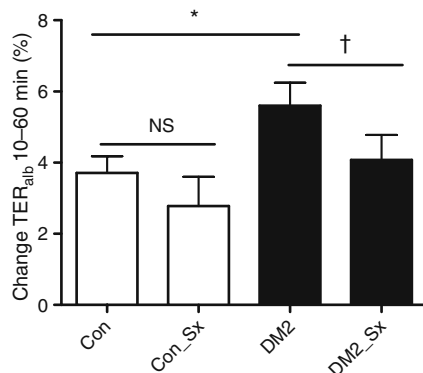


Fig. 5 The effect of sulodexide on vascular permeability in control and diabetic participants. Percentage change in TER_{alb} at baseline and after 8 weeks of treatment with sulodexide in controls ($n=10$; con and Con_Sx) and diabetic participants ($n=10$; DM2 and DM2_Sx); $p=NS$ for comparison of controls and controls following sulodexide treatment. Microvascular permeability was determined by the transcapillary escape rate of ^{125}I -labelled albumin (TER_{alb}) between 10 and 60 min after infusion. Data are presented as means \pm SD. $*p<0.05$, $^\dagger p=0.08$

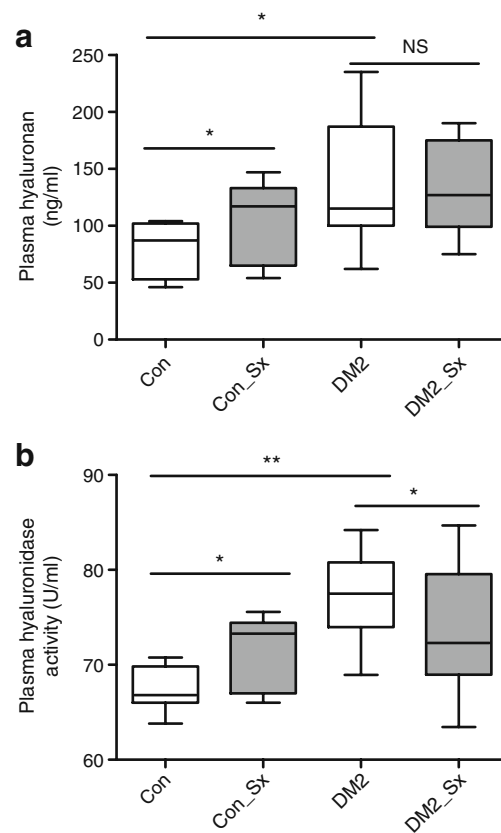


Fig. 6 The effect of sulodexide on plasma glycosaminoglycans in control and diabetic participants. **a** Circulating plasma hyaluronan levels at baseline and after 8 weeks of sulodexide administration in controls ($n=10$; con and Con_Sx, respectively) and diabetic participants ($n=10$; DM2 and DM2_Sx, respectively). **b** Hyaluronidase levels at baseline and after 8 weeks of sulodexide administration in controls ($n=10$; con and Con_Sx, respectively) and diabetic participants ($n=10$; DM2 and DM2_Sx, respectively), $p<0.05$ for comparison of controls and controls following sulodexide treatment; $p<0.01$ for comparison of controls, controls following sulodexide treatment and diabetes group before sulodexide treatment; $p<0.05$ for diabetes group before and after sulodexide treatment. Data are presented as whisker plots with median (interquartile range). $*p<0.05$, $**p<0.01$

in these trials were characterised by hypertension and microalbuminuria, indicating glomerular endothelial damage. Note that the microvascular endothelium may respond differently upon therapeutic intervention compared with glomerular endothelium (as determined by urinary albumin excretion), reflecting different pathogenic pathways [38–40]. In this respect, urinary albumin excretion in diabetes mellitus has been attributed predominantly to proximal tubular dysfunction [41, 42], which is independent from changes in glycoalyx properties. Therefore, the lack of a change in urinary albumin excretion in the aforementioned trials does not preclude the finding of improved glycoalyx properties in our normoalbuminuric patients. In addition, the time scales may profoundly affect the observed results. Thus, vascular barrier function restoration may follow at a

later stage when the endothelial glycocalyx is already restored [12, 13]. To definitively answer the question whether early intervention with sulodexide is able to prevent the development of diabetes-associated microvascular damage, a trial comparing sulodexide with placebo in newly diagnosed normotensive/normoalbuminuric patients with type 2 diabetes mellitus is warranted.

Mechanistically, measuring the impact of sulodexide on glycocalyx dimension and properties in health and disease is a challenge [15]. Endothelial glycocalyx thickness depends on the rate of synthesis, the rate of shedding as well as the circulating levels of GAG-degrading enzymes, including hyaluronidase and heparanase [43, 44]. Surprisingly, an increase in the sublingual glycocalyx dimension with a concomitant decrease in retinal glycocalyx was observed following sulodexide administration in controls. These data imply that endothelial responses are heterogeneous not only in patients with type 2 diabetes compared with controls, but also in various vascular beds [45]. Apparently, the endothelial glycocalyx dimension is more closely regulated in the retina than the sublingual microvessels [46]. The driving force behind glycocalyx synthesis is most likely determined by enhanced abundance of the precursors glucosamine, galactosamine and glucuronic acid. Following intestinal degradation of orally administered sulodexide, increased levels of GAG precursors may contribute to the increased plasma levels of hyaluronan and hyaluronidase in controls. In diabetes mellitus, however, GAG degradation is increased [10, 21]. In these patients, decreased plasma hyaluronidase activity may be attributed to the inhibition of enzymatic glycocalyx degradation by the (sulodexide-mediated) increase in substrate bioavailability [12]. In support, sulodexide has been hypothesised to inhibit heparanase (and possibly hyaluronidase) activity, both of which are involved in GAG catabolism [47]. Therefore, in line with the pioneering study by Potter et al. [46], future *in vitro* endothelial glycocalyx studies will have to focus on the effect of GAG supplementation on the regulation of GAG-degrading enzymes in different vascular endothelia (glomerular, capillary and retina).

This study has several limitations. First, the dose of sulodexide used was not validated by a formal dose-finding study. Previous studies have indicated that a treatment period as long as 6 months may be required to establish the maximal effect [48]. This delayed mode of action virtually precludes performing a meaningful dose-finding study. Therefore, we selected the 200 mg sulodexide dose based predominantly on previously published data [35]. These data have shown improvement in the endothelial barrier function using comparable concentrations of sulodexide *in vitro* [16]. Note that further dose escalation may also, at some point, be limited by the anticoagulant

effects of heparan sulphates, which could potentially lead to bleeding complications. Second, our present proof-of-concept study was rather small, with only type 2 diabetes patients without cardiovascular and/or proteinuric disease included. Whereas the findings are potentially interesting, they need further confirmation in a larger cohort as well as in type 2 diabetes patients with more severe stages of vascular complications.

In conclusion, individuals with type 2 diabetes mellitus are characterised by a clear perturbation of the endothelial glycocalyx layer, which is thought to reflect increased vascular vulnerability. Oral sulodexide administration improves endothelial glycocalyx dimension to the same extent in two different vascular beds (sublingual and retinal), most likely because of enhanced precursor abundance for GAG synthesis. Improvement of glycocalyx dimension coincided with a trend toward normalised systemic vascular permeability and GAG metabolism. Collectively, the present findings imply that restoration of endothelial glycocalyx in humans may be a promising target to attenuate vascular dysfunction in type 2 diabetes mellitus. Further research is required to evaluate which treatment modalities are most likely to establish improvement in endothelial glycocalyx dimension. More importantly, prospective studies should address whether the concept holds true that endothelial glycocalyx improvement predicts long-term cardiovascular benefit in diabetes mellitus.

Designations F. Holleman, M. Nieuwdorp, E.S.G. Stroes and H. Vink designed the study. L. N. Broekhuizen, B. A. Lemkes, H. L. Mooij, M. C. Meuwese and H. Verberne performed the research. R. O. Schlingemann, contributed the analytical tools (FAG). L. N. Broekhuizen, B. A. Lemkes, M. Nieuwdorp, E. S. G. Stroes and H. Vink analysed the data and drafted the paper. All authors critically reviewed the manuscript.

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Duality of interest All authors have no conflict of interest to declare. Alfa Wasserman provided the sulodexide, but was not involved in data analyses/interpretation or writing of the manuscript.

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