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The complement system plays a key role in the pathophysiology of kidney thrombotic microangiopathies (TMA), as illustrated by atypical hemolytic uremic syndrome. But complement abnormalities are not the only drivers of TMA lesions. Among other potential pathophysiological actors, we hypothesized that alteration of heparan sulfate (HS) in the endothelial glycocalyx could be important. To evaluate this, we analyzed clinical and histological features of kidney biopsies from a monocentric, retrospective cohort of 72 patients with TMA, particularly for HS integrity and markers of local complement activation. The role of heme (a major product of hemolysis) as an HS-degrading agent *in vitro*, and the impact of altering endothelial cell (ECs) HS on their ability to locally activate complement were studied. Compared with a positive control, glomerular HS staining was lower in 57 (79%) patients with TMA, moderately reduced in 20 (28%), and strongly reduced in 37 (51%) of these 57 cases. Strongly reduced HS density was significantly associated with both hemolysis at the time of biopsy and local complement activation (C3 and/or C5b-9 deposits). Using primary endothelial cells (HUVECs, Glomerular ECs), we observed decreased HS expression after short-term exposure to heme, and that artificial HS degradation by exposure to heparinase was associated with local complement activation. Further, prolonged exposure to heme modulated expression of several key genes of glycocalyx metabolism involved in coagulation regulation (*C5-EPI*, *HS6ST1*, *HS3ST1*). Thus, our study highlights the impact of hemolysis on the integrity of endothelial HS, both in patients and in endothelial cell models. Hence, acute alteration of HS may be a mechanism of heme-induced complement activation.

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Lay Summary

Thrombotic microangiopathies (TMAs) are a heterogeneous group of severe diseases with a particular kidney tropism. The pathophysiology of TMAs involves the complement system, but also possibly other players, such as endothelial glycocalyx. Our work provides evidence that hemolysis, a key feature of TMA, and in particular the released heme, is associated with the degradation of heparan sulfates (HS) of the glomerular glycocalyx on kidney biopsies of patients with TMA, as well as on endothelial cell models. This alteration increases cells' susceptibility to local complement activation, thus amplifying endothelial aggression, which drives TMA. This study highlights the impact of hemolysis on the integrity of endothelial HS, suggesting that acute alteration of endothelial glycocalyx is a mechanism of heme-induced complement activation.

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Thrombotic microangiopathy (TMA) is a nonspecific histologic lesion related to endothelial damage and characterized by fibrino-platelet thrombi in small-caliber vessels.[1] This lesion is the cornerstone of various diseases, including hemolytic uremic syndrome (HUS), a kidney-tropism TMA responsible for acute kidney injury

(AKI). Complement overactivation plays a key role in HUS, notably in the atypical form, in which 40%–60% of patients present with genetic or acquired abnormalities affecting the alternative pathway, and whose treatment is currently based on C5 inhibitors.[2] The variable incidence and symptoms of HUS, even for a given genetic abnormality,[3] underline the multifactorial nature of this disease. Factors other than complement overactivation must therefore combine to overwhelm the endothelial regulation of the complement (the "multiple-hits" theory) and participate in the occurrence of TMA lesions.[4,5] Among the key players in vascular homeostasis, and also able to modulate endothelial susceptibility to complement activation, is the glycocalyx. Its involvement in HUS and TMA lesions, although suggested,[6] remains poorly investigated.

The endothelial glycocalyx, a matrix rich in glycosaminoglycans, proteoglycans, and glycoproteins, lines the luminal pole of endothelial cells, forming their primary interface with circulating blood. It has several functions critical to the maintenance of a healthy vasculature: it interacts with many circulating components, regulates vascular permeability and mechanosensing, and has anti-inflammatory and antithrombotic properties.[6–8] Heparan sulfates (HS) constitute the majority of the glycosaminoglycans and modulate local complement activation by recruiting the complement regulatory protein factor H (FH), the major inhibitor of the alternative pathway.[9,10]

We here assessed the integrity of glomerular endothelial HS in a cohort of 72 patients with TMA. We describe an association between hemolysis, specifically at the time of biopsy, with HS degradation and local complement activation. In *in vitro* endothelial cell models (human umbilical vein [HUVECs] and glomerular [GECs] endothelial cells), we observed complement activation after artificial HS degradation, as well as heme-induced alteration of HS, independently of an impairment of the enzymatic processes required for HS formation. Thus, the disorganization of HS could be a mechanism of heme-induced complement activation and could participate in sensitization of endothelial cells to dysregulated complement.

METHODS

Patients with TMA

Patient selection. We retrospectively identified, from the digital records of the Department of Pathology, Lille University Hospital, the native kidney biopsy specimens with histologic TMA from patients treated between January 1, 2005, and December 31, 2020. Cases in which TMA lesions were satellite to another predominant glomerulonephritis were excluded, as were cases with no, or inadequate, tissue. Informed consent from each patient was previously obtained for the use of clinical data, remaining material for research purposes (DC 2008-642).

Kidney biopsies. Sections (4 μm thick), from formalin-fixed, paraffin-embedded tissue, were stained with Masson trichrome, periodic acid–Schiff, or Jones methenamine silver. Frozen sections (6 μm thick) were stained for immunofluorescence (IF) assays as part of the standard workup (e.g., C3 [Venta-Roche, 760-2686]). All biopsies were reviewed by 2 senior kidney pathologists (VG and JBG) without knowledge of the associated clinical information. Glomerular TMA was defined by the presence of thrombi, subendothelial edema, endothelial swelling, and/or mesangiolysis in glomeruli; and arteriolar TMA was defined by the presence of thrombi, subendothelial edema, myointimal proliferation, and/or inflammatory infiltrate in arterioles. Glomerulosclerosis, interstitial fibrosis, and tubular atrophy were evaluated as a percentage of total analyzed glomeruli or biopsy surface.

Immunolabeling of complement proteins. C3d staining was performed on formalin-fixed, paraffin-embedded sections (Ventana-Roche, 760-4522; 1/1; pH 8.4), and C5b-9 staining was performed on formalin-fixed, paraffin-embedded (clone B7; a gift from Paul Morgan, Cardiff, UK; 1/50; pH low) or frozen (Dako, M0777; 1/50) sections. The staining intensity was evaluated using a semi-quantitative scoring system (negative, 0; weak, 1+; moderate, 2+; and strong, 3+) where a staining intensity ≥2+ was considered to be positive.[11] Local complement activation was defined as C3 ≥2+ and/or C5b-9 ≥2+ in patients with at least 2 available stainings.

Immunolabeling of HS. Frozen sections were stained for HS (Amsbio, JM403, 370730, 5 μg/ml), then revealed with AF488 goat anti-mouse IgM (Invitrogen, A10680, 2 μg/ml). The whole slides were scanned with a Zeiss Axioscan Z1 (20×/numerical aperture, 0.8), and processed using the NIS-Elements AR software 3.2.11 (Nikon). Intensity was blindly evaluated by 2 independent operators (TL and VG) using a similar semiquantitative score to the above. The percentage of area fluorescent intensity of JM403-positive glomeruli, normalized to total number of glomeruli, was also quantified. Peritumoral kidney tissue served as a positive control (n = 4).

Clinical data

Hemolysis was defined by the concurrent measure of a haptoglobin < 0.5 g/L and lactate dehydrogenase > 500 IU/L. AKI was defined as a 1.5-fold increase of serum creatinine compared with the expected norm or the patients' last known value. Malignant hypertension was defined as diastolic arterial pressure >130 mm Hg associated with central nervous system, cardiac, retinal, or kidney injury.[12] Extrarenal events included acute cardiac (at least 2-fold increase of troponin I rate in the first 24 hours following admission), neurologic disorders (impaired alertness or focal deficit with posterior reversible encephalopathy syndrome lesions in magnetic resonance imaging), hepatic cytolysis, and concomitant diarrhea. The clinical features of each patient were independently reviewed by 2 nephrologists (TL [reviewer 1 {R1}] and MF [reviewer 2 {R2}]), enabling their attribution to 1 of 3 TMA groups: 1, HUS (requiring [i] the presence of both AKI and microangiopathic hemolytic anemia at the time of diagnosis and [ii] the determination of an underlying etiology consistent with the diagnosis of HUS, based on the Kidney Disease: Improving Global Outcomes [KDIGO][13,14]); 2, non-HUS acute TMA (aTMA; requiring [i] the presence of AKI and [ii] favorable outcome of AKI and/or hemolysis if present after blood pressure management); or 3, chronic TMA (cTMA; no identification of microangiopathic hemolytic anemia nor AKI). In case of disagreement, patients' data were reviewed by a third clinical nephrologist (CL [reviewer 3 {R3}]). Classification of patients was performed without knowledge of the immunostaining data.

Syndecan assays in human sera

The shedding of HS-core proteins syndecan-1 (SDC-1) and syndecan-4 (SDC-4) was assessed in hemolytic patients' sera (n = 4 [1 Shiga toxin–producing *Escherichia coli* HUS, 2 atypical HUS, and

1 aTMA]) and in healthy donors' sera (n = 12 [from the Etablissement Français du Sang]) by slot blot immunoassay. Samples were filtered under vacuum through a nitrocellulose membrane using a Bio-Dot SF apparatus (Bio-Rad). The membrane was then blocked for 1 hour at room temperature (RT) in Tris/Tween buffer (20 mM Tris/HCl, 150 mM NaCl, and 0.1% Tween 20, pH 7.6) supplemented with 5% bovine serum albumin. Incubation with mouse antibodies to SDC-1 (DL-101) and SDC-4 (5G9; Santa-Cruz Biotechnology) was performed overnight at 4 °C (1/1000) in Tris/Tween buffer supplemented with 3% bovine serum albumin. The membrane was then washed with Tris/Tween buffer and incubated with horseradish peroxidase–conjugated anti-mouse IgG (1/10,000) for 1 hour at RT. After washing, immunoreactive proteins were detected using electrochemiluminescence prime Western blotting detection (GE Healthcare). Quantification of immunostaining intensity was performed using ImageJ software.

Endothelial cell assays

Cell culture. Primary GECs (iCelltis; n = 4 donors) and HUVECs (n = 6 donors) were cultured with EGM2-MV (Lonza)[15] until passage 5 (GECs) and M199 20% fetal calf serum (Dutscher) 10% EGM2-EC (Lonza)[16,17] until passage 3 (HUVECs). Cells were exposed to the oxidized form of heme (hemin or ferriprotoporphyrin IX), designated as heme (Sigma-Aldrich, H9039) at indicated concentrations for 30 minutes at 37 °C in M199, or overnight in serum-free medium OPTI (Thermo Fisher).[17] To obtain an artificial degradation of HS, cells were treated with a combination of heparinase I, II, and III (Iduron) at concentrations of 0.2, 1, or 2 mU/10^6 cells (hereafter referred to as a "1X," "5X," and "10X," respectively), diluted in M199.[18] Normal human serum (NHS) was used as a source of complement (diluted 3× with M199 medium, $CaCl_2$ 1 mmol/L, $MgCl_2$ 1 mmol/L, for 30 minutes at 37 °C). Heat-treated NHS (decomplemented-NHS) was used as a negative control.

Flow cytometry. Cells were washed, nonenzymatically detached, labeled, and analyzed by flow cytometry (Fortessa X20).

A minimum of 10,000 cells *per* condition was required, and dead cells were excluded using LIVE/DEAD (L23105, ThermoFisher) reagent. The data were assessed using FlowJo software (V10.6.1; FlowJo LLC). The following antibodies were diluted in phosphate-buffered saline–bovine serum albumin 1% and incubated for 30 minutes at 4 °C: anti-HS (10E4, Amsbio, 370255, 20 μg/ml) (revealed by AF488 goat anti-mouse IgM [Invitrogen, A10680, 2 μg/ml]) and anti–C3c–fluorescein isothiocyanate (Dako, F020102-2, 30 μg/ml).

Immunofluorescence. Cells were washed and fixed with paraformaldehyde 4%, then labeled with anti-HS (10E4, Amsbio, 370255, 5 μg/ml; 16 hours, 4 °C) and anti–C3c–fluorescein isothiocyanate (Dako, F020102-2, 12 μg/ml; 1 hour, RT). Anti-HS was revealed by AF488 goat anti-mouse IgM (Invitrogen, A10680, 2 μg/ml) or AF555 goat anti-mouse IgM (Thermo Fisher, A28180, 2 μg/ml; 2 hours, 4 °C). After cells' exposure to anti–thrombin III (AT III; Abcam, AB62542, 10 μg/ml; 2 hours, RT),[19] AT III was stained with anti–AT III (Invitrogen, MA534940, 5 μg/ml; overnight, 4 °C) and revealed by AF647 goat anti-mouse IgG (Invitrogen, A-21235, 2 μg/ml; 1 hour, RT). Nuclei were stained with 4′,6-diamidino-2-phenylindole (Sigma, D9542, 2 μg/ml). The whole slides were scanned with a Zeiss Axioscan Z1 (20×/numerical aperture, 0.8), and percentage area of staining, normalized to nuclei number and expressed in μm^2 of positive areas, was quantified using ImageJ software (NIH, version 1.52, 2018). Illustrative photomicrographs were performed with confocal microscopes (Spinning Disk, Zeiss Axio Observer Z1–Yokogawa CSU-X1, and ZEISS CLSM 710).

Cell supernatant assays. After exposure to heme followed by a 30-minute incubation in NHS (37 °C), cell supernatants were collected and a slot blot immunoassay was used to assess the shedding of SDC-1 and SDC-4, as described above.

Reverse transcriptase with quantitative polymerase chain reaction (RT-qPCR). RNA extraction from HUVECs and GECs was performed with NucleoSpin RNA plus kit (Marcherey-Nagel). After standard reverse transcriptase polymerase chain reaction, amplification of transcripts of interest (*NDST1*, *NDST2*, *NDST3*, *HS2ST1*, *HS3ST1*, *HS3ST2*, *HS3ST3A1*, *HS3ST3B1*, *HS3ST5*, *HS6ST1*, *HS6ST2*, *C5-EPI*, and *EXT1)* was performed using specific primers[20] (Supplementary Table S1). The mean cycle threshold values for both the target and internal control (hypoxanthine-guanine phosphoribosyl transferase [*HPRT*]) were determined for each sample (in triplicate). The relative amplification was calculated according to the Pfaffl method, divided by each condition's *HPRT* expression.[21]

Statistical analyses

Statistical analysis was performed with R software (version 3.6.3.), using the "compareGroups 4.0" package, and GraphPad Prism software (version 9.3.0), also used for graphical representations. Patients' groups were compared with baseline variables by χ^2 or Fisher exact tests (categorical data) and Student's *t* tests or Mann-Whitney test (continuous data), according to the sample size and the distribution of the variables. When >2 groups were compared, 1- or 2-way analysis of variance tests were performed on continuous data, according to their respective distributions. Clinical results are presented as medians with interquartile ranges. Pearson correlation tests were used to compare semiquantitative and quantitative JM403 staining tests, with a significant relationship being retained at a value > 0.7. *In vitro* data were compared by 1-way analysis of variance or Dunn tests and are presented as means with SEM of the tested condition compared with the control. Distributions were assessed using the Shapiro-Wilk test. $P < 0.05$ was considered significant.

RESULTS

Clinical characteristics of patients with TMA

TMA was identified in 132 of 1484 (8.9%) native kidney biopsies performed in Lille CHU between 2005 and 2020. Sixty biopsies were excluded (details in Figure 1), resulting in a final cohort of 72 patients with a mean age at diagnosis of 41.4 years. Diagnoses of HUS, other aTMA, and cTMA were made in 21, 25, and 26 patients, respectively. Briefly, patients with HUS had a lower hemoglobin (8.3 [HUS] vs. 10.1 [aTMA] vs. 12.4 g/dl [cTMA]; $P < 0.001$) and platelet count (54 vs. 124 vs. 209 × 10^9/L; $P < 0.001$), a higher serum creatinine (5.8 vs. 3.7 vs. 1.7 mg/dl; $P < 0.001$), and greater prevalence of hematuria (100% vs. 65.2% vs. 56.2%; $P = 0.004$) or extrarenal events. Histologic lesions were also more pronounced in acute presentations (glomerular TMA: 90.5% [HUS] vs. 80% [aTMA] vs. 57.7% [cTMA; $P = 0.028$]; arteriolar TMA: 79% vs. 92% vs. 50% [$P = 0.003$]), as were complement deposits (C3 and/or C5b-9: 80% vs. 54% vs. 33%; $P = 0.011$). More details are given in Table 1 and in Supplementary Table S2.

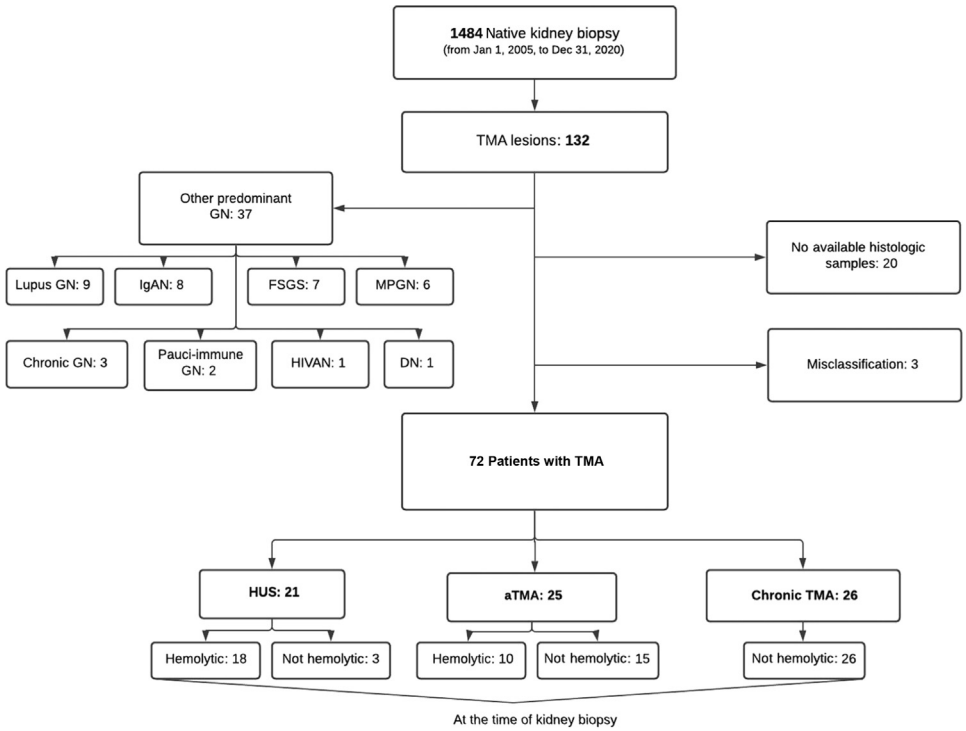

Figure 1 | Flowchart: exclusion criteria and final composition of the cohort. Classification of patients in hemolytic uremic syndrome (HUS), acute thrombotic microangiopathy (aTMA), or chronic thrombotic microangiopathy (TMA) groups was performed without knowledge of the immunostaining data by 2 or 3 (for the cases with disagreement) independent clinical nephrologists. DN, diabetic nephropathy; FSGS, focal segmental glomerular sclerosis; GN, glomerulonephritis; HIVAN, HIV-associated nephropathy; IgAN, IgA nephropathy; MPGN, membranoproliferative glomerulonephritis.

The decrease of glomerular HS density is associated with hemolysis in human kidney

The intensity of glomerular HS and complement deposits (C3 and C5b-9) were graded as shown in Figure 2a. Glomerular HS staining was strong (normal; 3+), moderate (2+), and low or negative (0–1+) in 15, 20, and 37 patients with TMA, respectively. Figure 2b shows that the semiquantitative analysis of nonroutine glomerular HS (GHS) staining was consistent with the quantitative percentage area fluorescent intensity analysis ($r = 0.78$). Patient characteristics according to the intensity of glomerular HS staining are summarized in Table 2. No difference was found in age, sex, or blood pressure among the groups with different staining intensities. Less-intense staining (0–1+) was significantly associated with hemolysis at diagnosis ($P = 0.037$), and this association was even stronger with hemolysis at the time of biopsy ($P = 0.008$). Consistently, median plasma lactate dehydrogenase levels were higher (505 [289–690] vs. 317 [250–351] IU/L; $P = 0.027$), and plasma haptoglobin was lower at the time of biopsy (0.3 [0.08–1.55] vs. 1.34 [0.18–2] g/L; $P = 0.15$) in the impaired-HS group (0–1+) compared with the preserved-HS group (2–3+; Figure 2c). Although serum creatinine at diagnosis was associated with less-intense GHS labeling ($P = 0.022$), no difference of HS staining was found for AKI ($P = 0.071$), ischemic acute lesions (glomerular ischemia, $P = 0.067$; or tubular necrosis, $P = 0.22$), or clinical diagnosis ($P = 0.219$). Indeed, GHS was impaired (0–1+) in 67% of patients with HUS, but also in 42% of patients with cTMA. Considering the GHS 0–1+ population, we noted a significantly higher glomerulosclerosis in nonhemolytic compared with hemolytic patients (39% ± 8% vs. 12% ± 4%; $P = 0.0058$); and in the group with cTMA, GHS 0–1+ patients had a more pronounced glomerulosclerosis than their GHS 2–3+ counterparts (Supplementary Figure S1). In line with these findings, subgroup analyzes showed that nonhemolytic patients with altered glycocalyx (0 or 1+) were significantly more associated with glomerulosclerosis (33%) than nonhemolytic patients with preserved glycocalyx (2 or 3+; 19%), or hemolytic patients with preserved glycocalyx (8%) or altered glycocalyx (14%; $P = 0.021$; Supplementary Table S3). No association was found between low GHS staining and etiology or prognosis (death or end-stage renal disease).

All analyses were also performed after exclusion of patients with cTMA. Hemolysis at the time of biopsy remained significantly associated with negative or low GHS staining ($P = 0.025$; Supplementary Table S4).

Hemolysis and altered GHS are associated with local complement activation in human kidney

Both altered GHS and hemolysis were significantly associated with local complement activation ($P < 0.001$), as defined by the presence of C3 and/or C5b-9 deposits in glomeruli. Significant C3 and C5b-9 deposits were respectively identified in 37% and 65% of GHS 0–1+ patients versus 19% ($P = 0.038$)

Table 1 | Patients' features according to clinical diagnosis

Variable	HUS (n = 21)	aTMA (n = 25)	cTMA (n = 26)	*P* value	n
Clinical features at presentation					
Age, yr	39.0 (30–59)	37 (32–48)	53.0 (35.2–63)	0.101	72
Sex ratio (F/M)	12:9	11:14	10:16	0.431	72
AKI	21 (100)	25 (100)	0 (0.00)	**<0.001**	72
CKD	1 (4.76)	7 (28.0)	17 (65.4)	**<0.001**	72
Requirement for dialysis	13 (61.9)	10 (40.0)	1 (3.85)	**<0.001**	72
Hemolysis at diagnosis	21 (100)	14 (56.0)	0 (0.00)	**<0.001**	71
Hemolysis at kidney biopsy	18 (85.7)	10 (40.0)	0 (0.00)	**<0.001**	69
Extrarenal events					
Elevated liver enzymes	5 (23.8)	5 (20.0)	0 (0.00)	**0.020**	72
Neurologic failure	9 (42.9)	9 (36.0)	0 (0.00)	**0.001**	72
Cardiologic failure	11 (52.4)	10 (40.0)	0 (0.00)	**<0.001**	72
Diarrhea	7 (33.3)	4 (16.0)	0 (0.00)	**0.003**	72
Biological features					
sCr, mg/dl	5.8 (2.7–7.2)	3.7 (2.5–5.0)	1.7 (1.3–2.4)	**<0.001**	72
Hemoglobin, g/dl	8.3 (8.1–9.7)	10.1 (9.4–11.2)	12.4 (10.6–13.0)	**<0.001**	71
Platelets, G/L	54 (22–86)	132 (86–213)	209 (155–260)	**<0.001**	71
Haptoglobin, g/L	0.08 (0.08–0.08)	0.16 (0.08–1.6)	1.83 (1.51–2.0)	**<0.001**	53
Schizocytes	18 (85.7)	9 (39.1)	0 (0.00)	**<0.001**	58
Low serum C3	3 (14.3)	0 (0.00)	0 (0.00)	0.053	62
Hyperleukocytosis	12 (60.0)	11 (44.0)	2 (7.70)	**0.001**	71
Proteinuria	19 (90.1)	22 (88.0)	23 (92.0)	1.000	71
Hematuria	19 (100)	15 (65.2)	13 (56.5)	**0.004**	65
Histologic features					
Glomerular TMA	19 (90.5)	20 (80.0)	15 (57.7)	**0.028**	72
Vascular TMA	15 (78.9)	23 (92.0)	12 (50.0)	**0.003**	68
Glomerular ischemia	16 (76.2)	22 (88)	13 (50)	**0.009**	72
Glomerulosclerosis, %	1.5 (0–12)	18 (9–23)	33 (16–63)	**<0.001**	69
Complement activation					
Glomerular C3 and/or C5b-9 ≥ 2+	16 (80.0)	13 (54.2)	7 (33.3)	**0.011**	65
Glomerular C3 ≥ 2+ (IF-IHC)	6 (30.0)	8 (32.0)	2 (9.50)	0.143	66
C3 IF ≥ 2+	1 (5.6)	1 (5.0)	1 (7.7)	1.000	51
C3d IHC ≥ 2+	6 (35.3)	7 (41.2)	1 (6.25)	0.051	50
Glomerular C5b-9 ≥ 2+ (IF-IHC)	13 (68.4)	9 (37.5)	7 (33.3)	0.052	64
C5b-9 IF ≥ 2+	7 (41.2)	2 (10.0)	4 (30.8)	0.084	50
C5b-9 IHC ≥ 2+	10 (83.3)	7 (50.0)	4 (33.3)	**0.043**	38
Associated conditions					
Malignant hypertension	0 (0.00)	17 (68.0)	0 (0.00)	**<0.001**	72
Malignancy	4 (19.0)	2 (8.00)	6 (23.1)	0.374	72
Drugs	3 (14.3)	1 (4.00)	15 (57.7)	**<0.001**	72
Autoimmune diseases	0 (0.00)	5 (20.0)	1 (3.85)	**0.034**	72
Pregnancy	4 (19.0)	0 (0.00)	3 (11.5)	0.070	72
STEC	1 (6.25)	0 (0.00)	0 (0.00)	–	27
Treatment					
Plasma exchange	15 (71.4)	3 (12.0)	0 (0.00)	**<0.001**	72
Eculizumab	12 (57.1)	3 (12.0)	0 (0.00)	**<0.001**	72
Cyclophosphamide	0 (0.00)	1 (4.00)	0 (0.00)	0.639	72
Rituximab	2 (9.52)	0 (0.00)	0 (0.00)	0.082	72
Corticosteroids	2 (9.52)	1 (4.00)	0 (0.00)	0.192	72
Last follow-up					
Follow-up, mo	22 (6–75)	30 (5–76)	29 (12–71)	0.913	72
sCr, mg/dl	1.2 (0.9–1.7)	2 (1.9–2.2)	1.5 (1.2–1.9)	0.104	60
ESRD	4 (19.0)	9 (36.0)	6 (23.1)	0.383	72
Death	4 (19.0)	6 (24.0)	3 (11.5)	0.511	72

aTMA, acute thrombotic microangiopathy; AKI, acute kidney injury; CKD, chronic kidney disease; cTMA, chronic thrombotic microangiopathy; ESRD, end-stage renal disease; F, female; HUS, hemolytic uremic syndrome; IF, immunofluorescence; IHC, immunohistochemistry; M, male; sCr, serum creatinine; STEC, Shiga toxin–producing *Escherichia coli*; TMA, thrombotic microangiopathy.
Data are given as median (interquartile range) or n (%) unless otherwise indicated.
Significant values ($P < 0.05$) appear in bold.

and 23% ($P = 0.002$) in GHS 2–3+ patients (Table 2 and Figure 2d). Considering the hemolytic status at the time of biopsy (Table 3 and Figure 2d), deposits of C3 occurred more frequently in hemolytic versus nonhemolytic patients (36% vs. 10.5%; $P = 0.033$), whereas C5b-9 deposits appeared less discriminative (57% vs. 37%; $P = 0.217$). All analyses were also

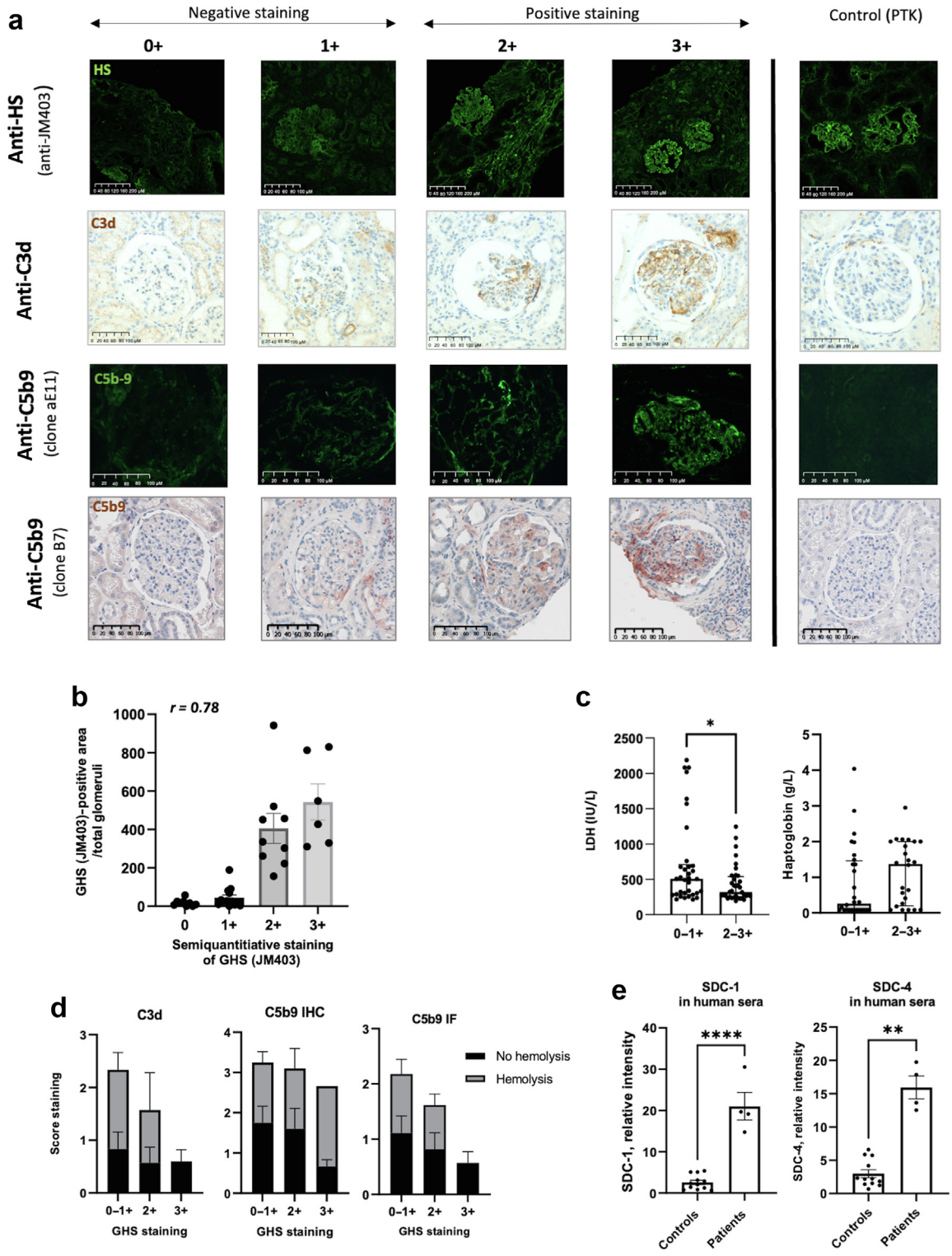

Figure 2 | Hemolysis at the time of biopsy is associated with decreased glomerular heparan sulfate (GHS) density and local complement activation in human native kidneys. (**a**) Representative pictures of labeling of glomerular heparan sulfate (HS; original magnification ×10), C3d, and C5b9 (clones B7 and aE11; original magnification ×20) in native kidney with thrombotic microangiopathy (TMA) lesions or in peritumoral kidney (PTK) used as control (right image). Staining intensity was assessed semiquantitatively as indicated (0, 1, 2, or 3+). (**b**) Correlation between quantitative and semiquantitative analysis of glomerular HS intensity staining (JM403). r = Pearson correlation coefficient. (**c**) Comparison of plasmatic lactate dehydrogenase (LDH; IU/L; left) and haptoglobin (g/L; right) levels (median ± interquartile range) according the intensity of glomerular HS staining (absent/low [0–1+] vs. mid/intense [2–3+]) at the time of kidney biopsy. *$P < 0.05$. (**d**) Staining score of C3d, C5b9 immunohistochemistry (IHC), and C5b9 immunofluorescence (IF; mean ± SEM) in glomeruli from TMA-kidney biopsies according to the intensity of GHS staining (absent/low: 0–1+; medium: 2+; and intense: [continued]

Table 2 | Patients' features according to HS staining

Variable	No or low (0–1+) (n = 37)	Mild (2+) (n = 20)	Strong (3+) (n = 15)	*P* value	n
Clinical diagnosis				0.219	72
HUS	14 (37.8)	5 (25.0)	2 (13.3)		
aTMA	12 (32.4)	9 (45.0)	4 (26.7)		
cTMA	11 (29.7)	6 (30.0)	9 (60.0)		
Clinical features					
Age, yr	36 (28–57)	50 (40–62)	38 (36–60)	0.079	72
Sex ratio, F/M	15:22	9:11	9:6	0.441	72
AKI	29 (78.4)	16 (80.0)	8 (53.3)	0.071	72
CKD	12 (32.4)	7 (35.0)	6 (40.0)	0.873	72
Hemolysis	21 (58.3)	11 (55.0)	3 (20.0)	**0.037**	71
Cardiologic event	15 (40.5)	2 (10.0)	4 (26.7)	**0.048**	72
Biological features					
sCr, mg/dl	3.7 (2.3–5.8)	3.0 (1.9–4.6)	1.7 (1.4–2.7)	**0.022**	72
Hemoglobin, g/dl	10.2 (8.3–12.0)	9.9 (8.3–11.8)	11.0 (10.2–12.6)	0.210	72
Platelets, G/L	130 (75–204)	110 (43–198)	217 (166–258)	**0.011**	71
Histologic features					
Hemolysis at KB	18 (52.9)	7 (35.0)	1 (6.67)	**0.008**	69
Glomerular TMA	29 (78.4)	15 (75.0)	10 (66.7)	0.630	72
Vascular TMA	28 (77.8)	14 (73.7)	8 (61.5)	0.539	68
Glomerular ischemia	24 (64.9)	18 (90.0)	9 (60.0)	0.067	72
Acute tubular necrosis, %	25 (0–40)	30 (2.5–50)	0 (0–22.5)	0.075	69
Glomerulosclerosis, %	16 (0–42.5)	17 (6.5–22)	21.5 (5–29.8)	0.691	69
Complement activation					
Glomerular C3 and/or C5b-9 ≥ 2+	27 (77.1)	7 (38.9)	2 (16.7)	**<0.0001**	65
Glomerular C3 ≥ 2+ (IF-IHC)	13 (37.1)	2 (10.5)	1 (8.30)	**0.038**	66
C3 IF ≥ 2+	3 (10.7)	0 (0.00)	0 (0.00)	0.509	51
C3d IHC ≥ 2+	11 (37.9)	2 (18.2)	1 (10.0)	0.176	50
Glomerular C5b-9 ≥ 2+ (IF-IHC)	22 (64.7)	6 (33.3)	1 (8.30)	**0.002**	64
C5b-9 IF ≥ 2+	11 (40.7)	2 (12.5)	0 (0.00)	**0.038**	50
C5b-9 IHC ≥ 2+	15 (75.0)	5 (62.5)	1 (10.0)	**0.003**	38

aTMA, acute thrombotic microangiopathy; AKI, acute kidney injury; CKD, chronic kidney disease; cTMA, chronic thrombotic microangiopathy; F, female; HS, heparan sulfate; HUS, hemolytic uremic syndrome; IF, immunofluorescence; IHC, immunohistochemistry; KB, kidney biopsy; M, male; sCr, serum creatinine; TMA, thrombotic microangiopathy.
Data are given as median (interquartile range) or n (%) unless otherwise indicated.
Significant values ($P < 0.05$) appear in bold.

performed after exclusion of patients with cTMA. Hemolysis at the time of biopsy remained significantly associated with local complement activation ($P = 0.05$; Supplementary Table S5).

Glycocalyx degradation products are increased in the serum of hemolytic patients with TMA

To evaluate the glycocalyx degradation under hemolytic conditions in humans, we assessed the serum levels of soluble, shedded HS core proteins SDC-1 and SDC-4 in hemolytic patients with TMA. Compared with healthy control serum, the relative intensity of both SDC-1 and SDC-4 was significantly elevated: the mean relative intensity of SDC-1 was 2.6 ± 0.5 in controls and 21.0 ± 3.4 in patients ($P < 0.0001$), and that of SDC-4 was 3.0 ± 0.6 in controls and 15.9 ± 1.7 in patients ($P = 0.0011$; Figure 2e).

Short-term exposure to heme alters endothelial HS density

To assess the impact of heme on HS *in vitro*, we performed a labeling of HS after incubation of GECs with increasing concentrations of heme. Under basal conditions, we observed a reticular HS labeling in IF (Figure 3a). Short-term exposure to heme was associated with a dose-dependent decrease of 10E4 HS label. Compared with controls, the detection of 10E4-HS decreased on average by ∼85% in IF ($P = 0.0013$) and in flow cytometry ($P = 0.011$) at 100 μM of heme (Figure 3b and c). Under short-term exposure to heme, we observed a dose-dependent increase in the release of soluble core proteins of SDC-1 and SDC-4 to the supernatant of the GECs. The relative intensity of both these HS-bearing proteoglycans was elevated compared with controls by, on average, ∼15.5 ± 201 ($P = 0.0024$) and ∼20.6 ± 2.3 ($P = 0.0008$), respectively, at 100 μM heme (Figure 3d), supporting our working hypothesis of the negative impact of heme exposure on HS integrity. Similar results were observed in HUVECs (Supplementary Figure S2).

The artificially induced degradation of endothelial HS makes endothelial cells more susceptible to complement activation *in vitro*

To study the direct impact of HS loss on the ability of endothelial cell membranes to locally activate the complement, we

Figure 2 | [continued] 3+) in hemolytic (gray) versus nonhemolytic (black) patients. (**e**) Syndecan-1 (SDC-1) and syndecan-4 (SDC-4) HS core-protein levels (mean ± SEM) in healthy controls (n = 12) or hemolytic TMA patients' sera (n = 4). **$P < 0.001$, ****$P < 0.0001$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Table 3 | Patients' features according to hemolysis at the time of KB

Variable	No hemolysis at KB (n = 43)	Hemolysis at KB (n = 26)	*P* value	n
GHS JM403 staining			**0.008**	69
0–1+	16 (37.2)	18 (69.2)		
2+	13 (30.2)	7 (26.9)		
3+	14 (32.6)	1 (3.85)		
Complement activation				
Glomerular C3 and/or C5b-9 ≥ 2+	15 (39.5)	18 (75.0)	**0.014**	62
Glomerular C3 ≥ 2+	4 (10.5)	9 (36.0)	**0.033**	63
C3 IF ≥ 2+	1 (4.00)	1 (4.35)	1.000	48
C3d IHC ≥ 2+	3 (10.7)	9 (47.4)	**0.007**	47
Glomerular C5b-9 ≥ 2+	14 (36.8)	13 (56.5)	0.217	61
C5b-9 IF ≥ 2+	6 (22.2)	6 (30.0)	0.790	47
C5b-9 IHC ≥ 2+	9 (40.9)	10 (71.4)	0.431	36

GHS, glomerular heparan sulfate; IF, immunofluorescence; IHC, immunohistochemistry; KB, kidney biopsy.
Data are given as n (%).
Significant values ($P < 0.05$) appear in bold.

developed an artificial enzymatic digestion of HS. After a short exposure to 3 different concentrations of a heparinase mixture, IF assays detected a significant dose-dependent reduction of 10E4-labeled HS staining of ∼80% after treatment with the 1X mix ($P = 0.0064$) and ∼95% with 5X ($P = 0.0005$) and 10X ($P = 0.0003$) compared with controls (Figure 4a). Similar results were observed by flow cytometry, with a respective mean reduction of ∼70% and ∼90% after treatment with the 1X ($P = 0.008$) and 5X ($P = 0.0002$) heparinase mixtures, respectively, compared with controls (Figure 4b). Complement activation was then studied in cells exposed to the 5X heparinase mixture or 100 μM heme, this last being a known inducer of complement in endothelial cells (positive control[22]). Using IF, C3b deposits increased respectively ∼5- and 3-fold under 100 μM heme or the 5X heparinase mix compared with controls, and we noted a concomitant decrease in 10E4-HS labeling (Figure 4c). Heparinase-induced increase of C3b deposition was dose dependent (Supplementary Figure S3). Finally, using heat-treated NHS (decomplemented serum) as a negative control, C3b deposition dropped compared with NHS cells treated under the same conditions (Figure 4d). Similar results were observed using flow cytometry (Figure 4e). Comparable results in HUVECs exposed to these same concentrations of heme and heparinases are shown in Supplementary Figure S2.

Long-term exposure to heme is associated with HS remodeling in HUVECs and to a lesser extent in GECs

To assess the effects of heme on HS biosynthesis, we measured the gene expression of the main enzymes involved in HS biosynthesis after a 16-hour exposure of HUVECs and GECs to increasing concentrations of heme. The expression profiles between basal condition (0) and exposure to 50 μM of heme of both cell types are shown in Supplementary Figure S4. *NDST1* exhibited significant basal expression, which remained stable under different test conditions, indicating a stable HS synthesis in both HUVECs and GECs (Figure 5a). Three enzymes implicated in the "anti-thrombin pattern" generation (Figure 5b) showed significant dose-dependent variations in HUVECs: compared with controls, *C5-EPI* expression was decreased by a factor of ∼0.38 ($P = 0.042$), *HS6ST1* expression was increased ∼1.6-fold ($P = 0.013$), and *HS3ST1* was increased ∼10-fold ($P = 0.002$) at 50 μM of heme (Figure 5a). Under the same conditions in GECs, *HS3ST1* was increased ∼6-fold ($P = 0.018$) but neither *C5-EPI* nor *HS6ST1* expression was significantly modulated (Figure 5a).

Exposure to heme is associated with modulation of HUVECs' and GECs' ability to bind AT III

To evaluate the phenotypic impact of these transcriptomic modifications of HS observed here, we tested the ability of HUVECs and GECs to bind AT III after heme exposure. Under basal conditions, we observed an AT III labeling in IF for both cells (Figure 5c). After 30 minutes' exposure to increasing doses of heme, we observed a global decrease of AT III staining in both cell types, consistent with global HS disruption induced by short-term exposure to heme (Figure 5d). After 16 hours' heme exposure, AT III staining increased by ∼2-fold compared with controls in HUVECs exposed to 25 and 50 μM of heme (Figure 5e), whereas no change was observed on the GECs.

DISCUSSION

This study describes the clinical and histologic features of a monocentric, retrospective TMA cohort, with a novel focus on HS, the main components of the endothelial glycocalyx. Lower glomerular HS density in human kidney biopsies was associated with hemolysis as well as local complement activation. Using a glomerular endothelial cell model, we established that short-term heme exposure altered HS density, and that artificial HS degradation induced complement activation. Finally, long-term exposure to heme modulated the gene expression of important enzymes implicated in both cell types' HS reshaping.

The involvement of HS in the regulation of the alternative pathway has already been demonstrated *in vitro*,[10] but this is the first report, to our knowledge, of reduced glomerular HS density in a clinical cohort of patients with TMA. The JM403-labeled HS has been studied in other kidney diseases, and a decrease of glomerular basement membrane HS was described in minimal change disease (3 patients), lupus nephritis (13 patients), or diabetic glomerulosclerosis, whereas the HS staining was conserved in IgA nephropathy (3 patients).[23,24] Given the possible competitive risk for glycocalyx degradation, our analysis excluded all patients for whom the TMA was satellite to a predominant glomerulonephritis. Within the TMA cohort studied here, JM403-labeled HS varied from null (no HS) to intense (preserved HS). The intensity of HS labeling did not appear to be affected by age, sex, or preexisting comorbidities, such as chronic kidney disease, although an impact of aging or uremic toxins on the

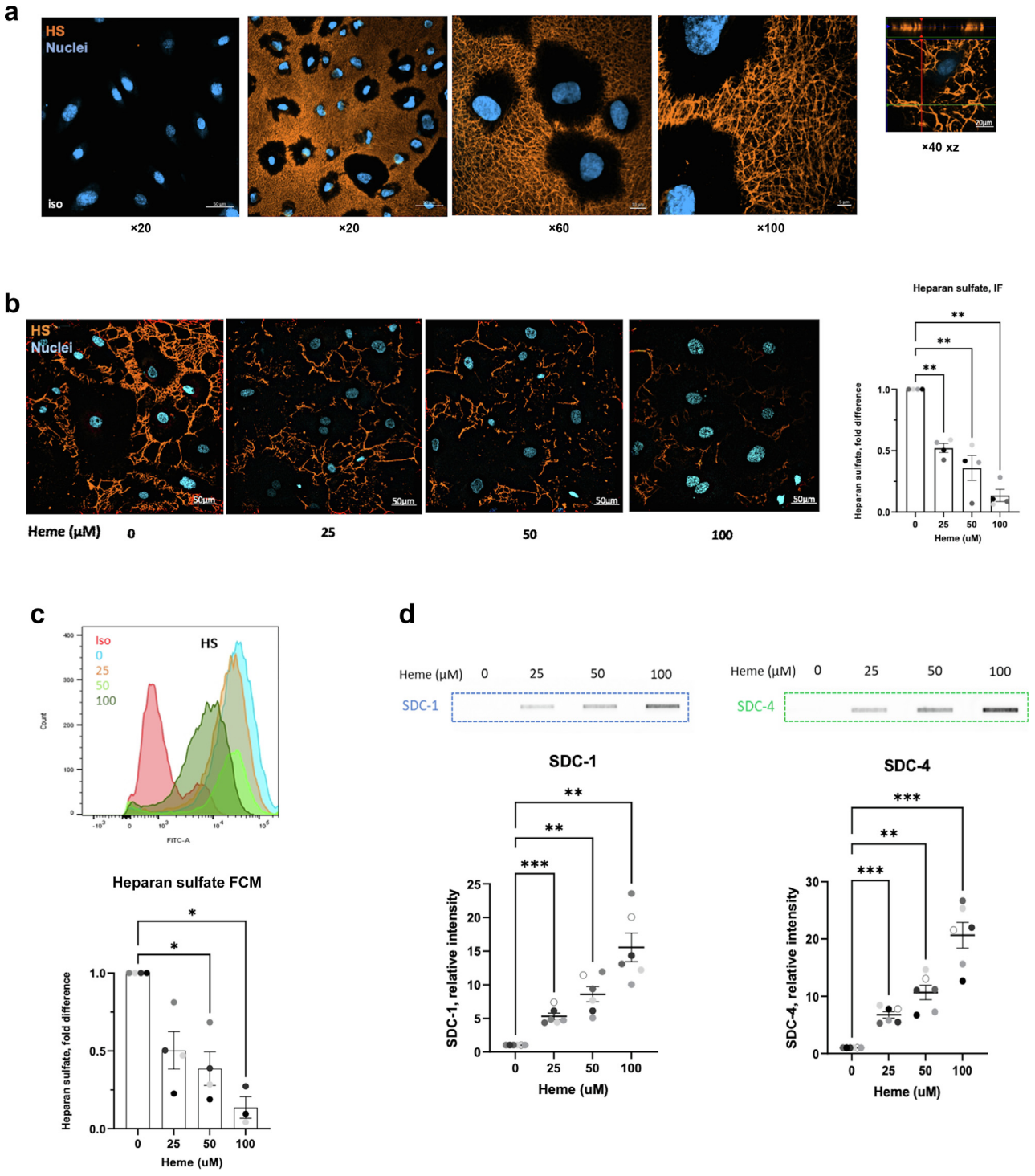

Figure 3 | Heme exposure is associated with heparan sulfate (HS) degradation on glomerular endothelial cells (GECs). (**a**) Representative pictures of glycocalyx HS staining (orange) at original magnification ×20, original magnification ×40, and original magnification ×64 focus, with xz plan reconstruction (original magnification ×40) on GECs by immunofluorescence (IF). Left picture corresponds to isotype (Iso) labeling. Cellular nuclei are 4′,6-diamidino-2-phenylindole (DAPI) labeled (blue). (**b**) Glycocalyx HS staining (orange) on GECs under basal condition (0) or with increasing concentration of heme (25, 50, or 100 μmol/L) by IF (original magnification ×20). Cellular nuclei are DAPI labeled (blue). Representative histograms of 4 independent experiments (mean ± SEM). $^{**}P < 0.01$. (**c**) HS expression on GECs under basal conditions (blue) or after exposure to 25 (orange), 50 (light green), or 100 μmol/L (dark green) of heme studied by flow cytometry (FCM). Isotype control appears as a red histogram (Iso). Representative graph of 4 independent experiments (mean ± SEM). $^{*}P < 0.05$. (**d**) Syndecan-1 (SDC-1) and syndecan-4 (SDC-4) HS core proteins released in GECs' supernatant after exposure to increasing heme concentration (0, 25, 50, or 100 μmol/L). Representative pictures of 6 independent experiments (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

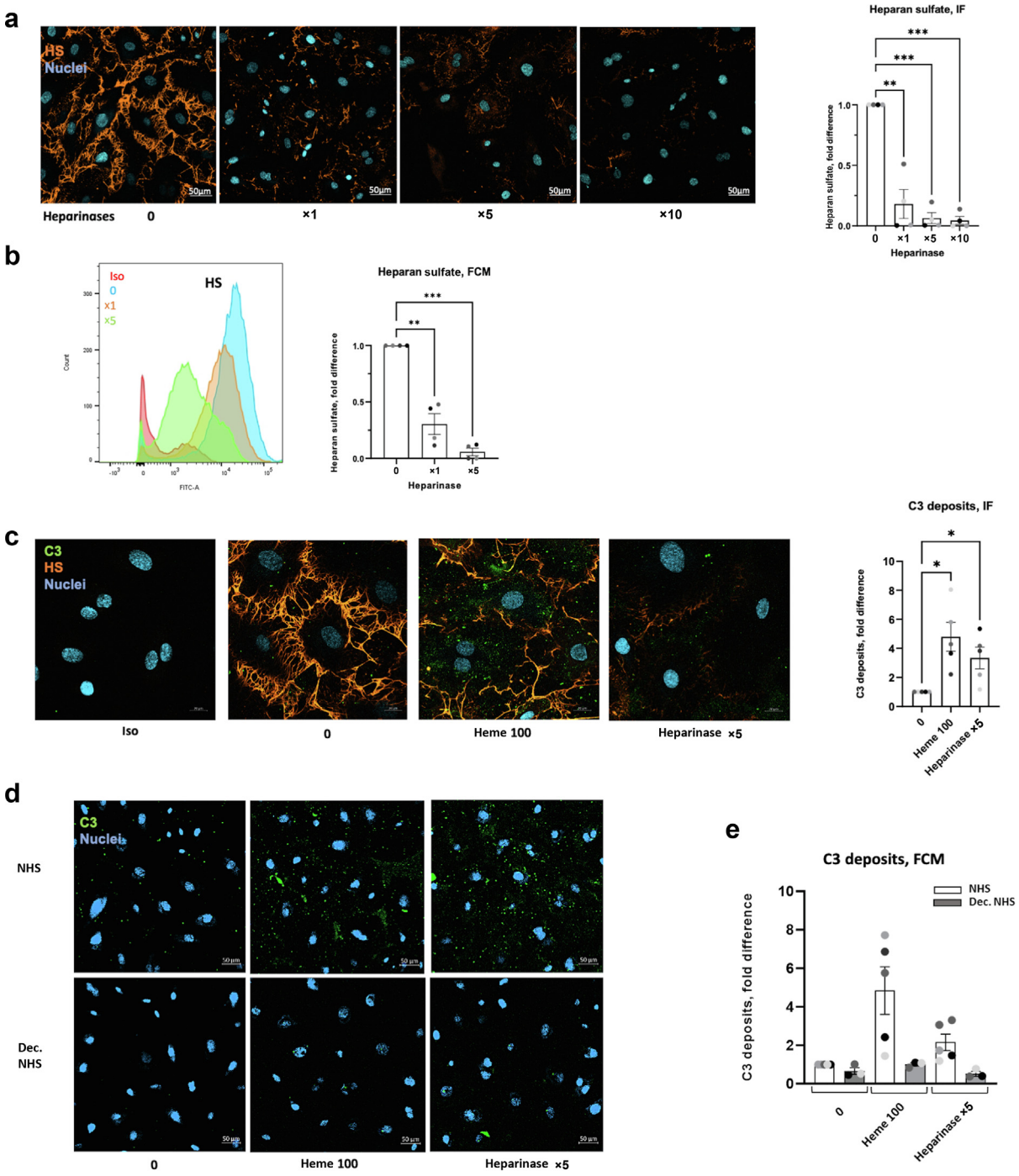

Figure 4| Artificial heparinase-mediated heparan sulfate (HS) degradation is associated with local complement activation on glomerular endothelial cells (GECs). (**a,b**) Glycocalyx HS staining on GECs treated with medium only (0) or with increasing concentrations of heparinases I + II + III (1X: 0.2 mU/10^6 cells; 5X: 1 mU/10^6 cells; or 10X: 2 mU/10^6 cells) studied by (**a**) immunofluorescence (IF; orange; original magnification ×20) and (**b**) flow cytometry (FCM). Cellular nuclei are 4′,6-diamidino-2-phenylindole (DAPI) labeled (blue). Representative images of 4 independent experiments (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$. (**c**) C3b fragment deposits (green) and HS staining (orange) in GECs treated with medium (0), heme 100 µmol/L, or heparinases I + II + III (5X) studied by IF (original magnification ×60). Cellular nuclei are DAPI labeled (blue). Representative histogram of 5 independent experiments (mean ± SEM). $^{*}P < 0.05$. (**d,e**) C3b fragment deposits in GECs treated with normal human serum (NHS) or decomplemented NHS (Dec. NHS) after treatment with medium (0), heme 100 µmol/L, or heparinases I + II + III (5X) studied by (**d**) IF (green; original magnification ×20) and (**e**) FCM (representative images of 3–5 experiments). Cellular nuclei are DAPI labeled (blue). Iso, isotype. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

glycocalyx has been suggested by others.[25–27] Low HS labeling was associated with several criteria for active TMA (hemolysis, low platelets, and high lactate dehydrogenase levels) and severity (elevated plasma creatinine or occurrence of a cardiovascular event). Low-intensity JM403 staining was thus more common in the HUS group, but no statistical association was found, and the clinical diagnosis (HUS, aTMA, or cTMA) failed to predict HS deterioration. This is partly related to the frequency of GHS 0–1+ status in patients with cTMA (42%), where we noted in this specific population that severe glomerulosclerosis was more common. In fact, subgroup analysis eventually demonstrated that patients without hemolysis but with altered GHS exhibited significantly more glomerulosclerosis than the others. Although we studied only permeable glomeruli, we cannot exclude the possibility that hyperfiltration of the remaining permeable glomeruli in glomerulosclerotic biopsies could alter the labeling.

Low-intensity HS labeling was associated with the presence of glomerular complement deposits. This is a novel finding in human kidneys, but similar results have been reported in mouse models. Eto *et al.* described glomerular HS degradation and C3 deposits in an anti–endothelial cell antibody–induced kidney–TMA model, both of which were prevented by intra-arterial perfusion of synthetic glycosaminoglycans.[28] In a kidney ischemia-reperfusion model, loss of glycocalyx and complement activation were counteracted by anti-C5 therapy,[29] suggesting that complement activation itself may induce glycocalyx damage. Local complement activation could also be explained by defective regulation. Circulating FH anchors itself to host cells through binding of its short consensus repeats (SCR) 7 and SCR 19–20 domains to endothelial glycocalyx.[30,31] Thus, 19–20-mutated FH failed to bind HS to glomerular endothelial cells *in vitro*,[32] and in patients with atypical HUS, FH abnormalities mainly affect the SCR 19–20 domains.[33] In any case, our results indicate that the integrity of endothelial HS is crucial for cells' protection against complement activation.

Hemolysis at the time of biopsy was strongly associated with lower GHS, suggesting a role for hemolysis-derived products, such as free heme, in endothelial glycocalyx injury. Physiologically sequestered in red blood cells, heme is the oxygen-binding prosthetic group of hemoglobin. In case of release during massive hemolysis, this porphyrin molecule bound to a ferric ion constitutes a powerful endothelial aggressor, promoting prothrombotic and proinflammatory effects, with complement activation among them.[5] Here, we report a novel mechanism of heme toxicity in endothelial cells. In our models, heme-induced HS degradation occurred after short-term exposure, which excludes *in vivo* modulation of HS-degrading enzyme genes, such as heparanases. Heparanases are stored in late endosomes as proheparanases, and their activation requires the cleavage of a linker peptide (rapid posttranslational regulation).[34] The HS degradation after only short-term exposure to heme could be explained by a heme-dependent cleavage of these proheparanases. We also observed that exposure of HUVECs and GECs to heme induced a release of SDC-1 and SDC-4 core proteins to the supernatant. It has been reported in a primary astrocyte model that heme induced matrix metallopeptidase 9 activity in a toll-like receptor 2–dependent manner.[35] Matrix metallopeptidase 9 could potentially act as a sheddase and alter endothelial HS integrity *via* the cleavage of HS proteoglycans from the cell surface.[36] Finally, another hypothesis is direct heme toxicity toward the polysaccharide chain. Free heme is a major provider of reactive oxygen species,[5] such as Fe^{4+}, H_2O_2, O_2^-, and NO_3^-. Rees *et al.* showed *in vitro* that another reactive oxygen species, hypochlorous acid, was able to depolymerize the HS chain.[37] In an Adriamycin (doxorubicin)–induced nephropathy mouse model, increased reactive oxygen species production was associated with depolymerization of HS and increased albuminuria. The addition of metal ions, such as Fe^{3+}, catalyzed this reaction.[38] Hemolysis-induced glycocalyx HS degradation could, therefore, be a novel mechanism contributing to local complement activation on endothelial cell membranes.

Because the half-life of HS is 3 to 4 hours, it can be assumed that overnight exposure to heme could phenotypically lead to significant remodeling of the expressed HS. We observed dose-dependent modulation for 3 enzymes important to HS biogenesis in heme-exposed HUVECs. HS3ST1 is the key enzyme for generation of the "anti-thrombin signature" (GlcA-GlcNS,3S,6S).[39] This specific pattern is the key determinant of HS and heparin required to activate AT III, the main circulating anticoagulant. Heme could therefore lead to the reshaping of endothelial cells' HS to give them an anticoagulant power,[40] which accords with the well-described dual nature of heme.[17] However, HS3ST1 can only catalyze the 3-*O*-sulfation of bioavailable (GlcA-GlcNS,6S) disaccharides, themselves generated by the upstream 6-*O*-sulfation of an N-sulfated glucosamine (GlcNS) linked to a *nonisomerized* glucuronic acid (GlcA), a reaction that can be catalyzed by HS6ST1.[41] As C5-epimerase isomerizes (GlcA) into iduronic acid (IdoA), (GlcA-GlcNS) bioavailability therefore crucially depends on the downregulation of *C5-EPI*.[41] Compared with HUVECs, GECs did not modulate *C5-EPI* and *HS6ST1* expression on exposure to heme. We may hypothesize that, if both endothelial cell types tend to counter the prothrombotic state induced by heme through upregulating their *HS3ST1*, the lack of upstream upregulation of *HS6ST1* and upstream downregulation of *C5-EPI* in GECs will generate less of the (GlcA-GlcNS,6S) substrate for upregulation of their HS3ST1, thus limiting their ability to generate an anti-thrombin pattern. These results are also in line with the somewhat lower AT III binding ability of GECs compared with HUVECs, observed here after long-term heme exposure, which could thus be interpreted as a poorer adaptation to heme-induced thrombotic stress. GECs have previously exhibited less adaptive capability compared with HUVECs, failing to increase *HO-1* expression after long-term exposure to heme.[17] Similarly, the lack of HS remodeling in heme-exposed GECs could contribute to the particular vulnerability of the kidney to hemolysis and complement activation, in line with the relative inability of GECs to bind FH compared with HUVECs.[17]

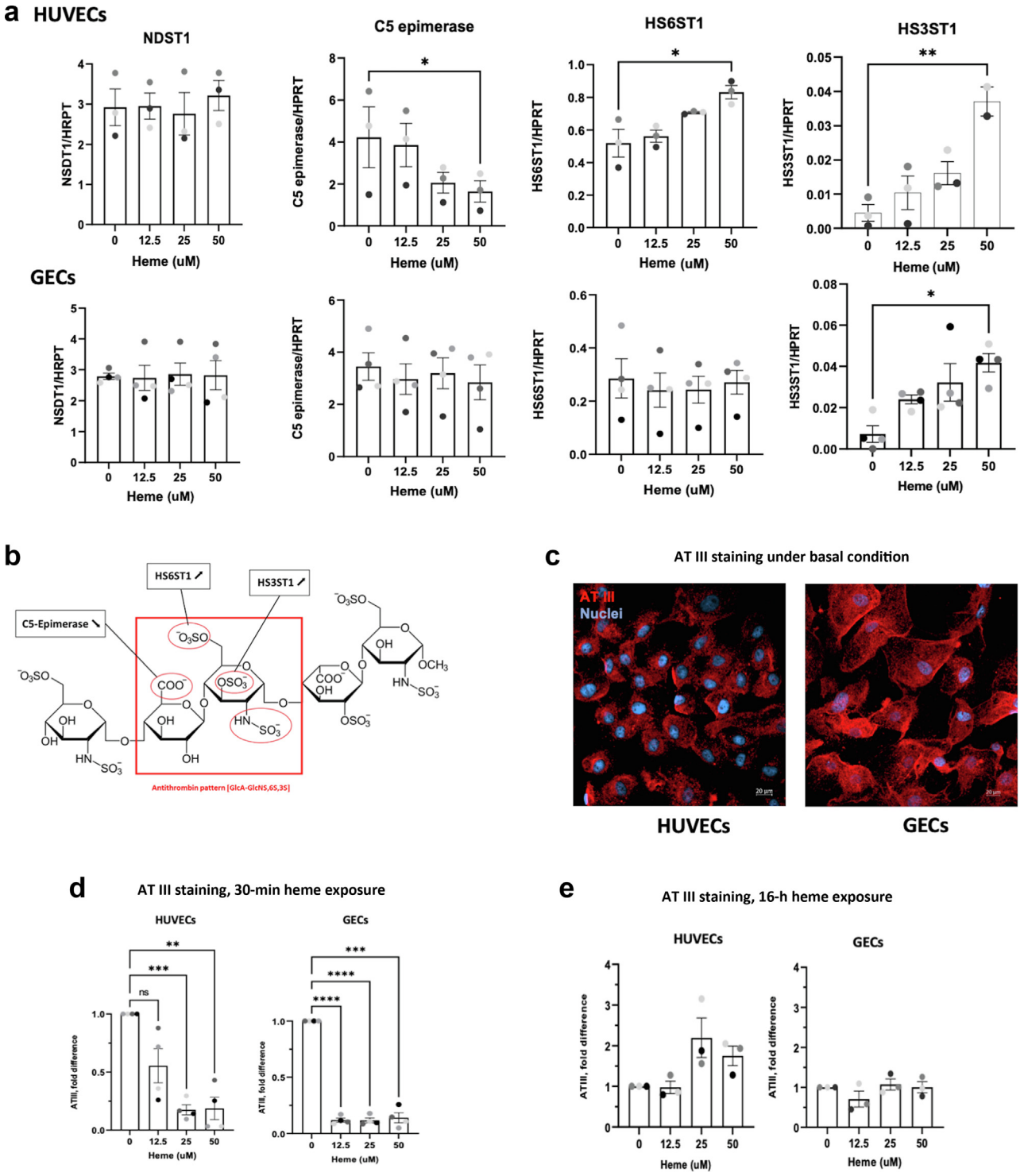

Figure 5 | Exposure to heme is associated with heparan sulfate (HS) reshaping and with modulation of human umbilical vein endothelial cells' (HUVECs') and glomerular endothelial cells' (GECs') ability to bind anti–thrombin III (AT III). (**a**) Gene expression of *NDST1*, *C5-EPI*, *HS6ST1*, and *HS3ST1*, enzymes implicated in HS biosynthesis, in basal condition (0) or after 16 hours' exposure to 12.5, 25, or 50 μmol/L of heme in HUVECs (above) and GECs (below). Results are presented as a ratio of the expression of the *HPRT* housekeeping gene (HUVECs: n = 3; GECs: n = 4). (**b**) Schematic Haworth representation of heparan sulfate/heparin's anti-thrombin pattern (GlcA-GlcNS,3S,6S) showing the 3 enzymes (C5-epimerase [C5-EPI], heparan sulfate 6-O-sulfotransferase 1 [HS6ST1], and heparan sulfate 3-O-sulfotransferase 1 [HS3ST1]) involved in its biosynthesis. (**c**) Representative photomicrographs of AT III binding (red) on HUVECs (left) or GECs (right) under basal condition in immunofluorescence (IF; original magnification ×20, confocal). Cellular nuclei are 4′,6-diamidino-2-phenylindole labeled (blue). (**d**) Representative histogram of independent experiments of AT III binding on HUVECs (left) or GECs (right) under basal condition (0) or after 30 minutes' exposure (n = 4) or (**e**) 16 hours' exposure of increasing concentrations (12.5, 25, and 50 μmol/L) of heme (n = 3) in IF (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$, $^{****}P < 0.0001$. HPRT, hypoxanthin-guantin phosphoribosyltransferase; NDST1, N-desacetylase and N-sulfotransferase 1. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

The impact of heme on HS integrity, and its ability to locally activate complement on endothelial cells, highlighted in our TMA-focused study, could be extrapolated to other hemolytic disease. Sickle-cell disease (SCD), a prototypic model of intravascular hemolysis, has been associated with elevated plasma sC5b-9, a reliable marker of complement terminal activation.[42] In the same study, HUVEC exposure to SCD sera was associated with *in vitro* C3b deposition, which was prevented by the heme-scavenger hemopexin, emphasizing the crucial role of heme in complement activation in SCD. Interestingly, van Beers *et al.* reported a decrease of glycocalyx thickening in 20 patients with SCD compared with healthy controls.[43] One could hypothesize that heme released in SCD during acute intravascular hemolysis could lead, as in our study, to endovascular glycocalyx disruption, with this loss itself enhancing local complement activation. These hypotheses deserve to be tested in future studies.

In summary, this study has highlighted the impact of hemolysis on the integrity of endothelial glycocalyx HS, both *in vivo* in kidney biopsies of patients with TMA, and in an *in vitro* glomerular endothelial cell model. We hypothesize that free heme released during intravascular hemolysis in HUS, or other contexts characterized by acute TMA, may induce an endothelial HS degradation, and that the loss of HS in itself potentializes the ability of endothelial membranes to locally activate the complement, thereby further increasing endothelial injury, and finally rendering the disease self-sustaining. Specific therapies targeting the regeneration or preservation of the endothelial glycocalyx, such as sulodexide or atrasentan, an endothelin-A receptor antagonist,[44,45] may therefore contribute to the control of these diseases, in addition to anti-complement therapy in atypical HUS, perhaps enabling less-frequent administration or cessation of this gold standard, but costly, therapy.

DISCLOSURE

All the authors declared no competing interests.

DATA STATEMENT

The data sets generated during and/or analyzed during the current study are available from the corresponding authors on reasonable request.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

TL, MM, FA, VG, and MF conceptualized the study. TL, FA, J-BG, AD, GG, CL, VG, and MF acquired the data. TL, MM, BL, and MF analyzed the statistics. TL and MF drafted the manuscript. All authors provided critical revision for important intellectual content and manuscript approval.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Table S1. Main characteristics and sequences of quantitative polymerase chain reaction (qPCR) primers.

Supplementary Table S2. Patients' clinical classification.

Supplementary Table S3. Patients' histologic features according to both hemolysis at time of kidney biopsy and glomerular heparan sulfate staining.

Supplementary Table S4. Patients' features according to heparan sulfate (HS) staining (*after exclusion of chronic thrombotic microangiopathy [cTMA]*).

Supplementary Table S5. Patients' features of according to hemolysis at the time of kidney biopsy (*after exclusion of chronic thrombotic microangiopathy [cTMA]*).

Supplementary Figure S1. Glomerulosclerosis is associated with impaired glomerular heparan sulfate (HS) staining. (**A**) Left: Glomerular HS staining (absent/low: 0–1+: red; mid: 2+: light green; strong: 3+: dark green) according to hemolysis, defined by low haptoglobinemia (y) and lactate dehydrogenase (LDH) elevation (x). Right: Glomerulosclerosis percentage (mean ± SEM) in hemolytic versus nonhemolytic patients at the time of kidney biopsy. $^{**}P < 0.01$. (**B**) Glomerulosclerosis percentage (mean ± SEM) in low (0–1+) versus high (2–3+) glomerular HS staining $^{**}P < 0.01$.

Supplementary Figure S2. Heme exposure is associated with heparan sulfate (HS) degradation, and artificial heparinase-mediated HS degradation is associated with local complement activation on human umbilical vein endothelial cells (HUVECs). (**A**) Glycocalyx HS staining on HUVECs under basal condition (0) or with increasing concentration of heme (25, 50, or 100 μmol/L) assessed by immunofluorescence (IF) (left) and flow cytometry (FCM) (right), fold difference (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$. (**B**) Syndecan-1 (SDC-1) and syndecan-4 (SDC-4) HS core protein released in HUVECs' supernatant after exposure to increasing concentrations of heme (0, 25, 50, or 100 μmol/L), fold difference (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$. (**C**) Glycocalyx HS staining on HUVECs treated with medium only (0) or with increasing concentrations of heparinases I + II + III (1X: 0.2 mU/10^6 cells; 5X: 1 mU/10^6 cells; or 10X: 2 mU/10^6 cells) studied by IF (left) and FCM (right), fold difference (mean ± SEM). $^{**}P < 0.01$, $^{***}P < 0.001$. (**D**) C3b deposits on HUVECs under basal conditions or after exposure to heme, 100 μmol/L, or heparinases I + II + III (5X), assessed by IF (left) and FCM (right) (mean ± SEM). $^{*}P < 0.05$, $^{**}P < 0.01$. (**E**) C3b fragment deposits (green) and HS expression (orange) in HUVECs treated with medium (0) or heparinases I + II + III (5X or 10X) studied by IF (×20). Cell nuclei are 4′,6-diamidino-2-phenylindole (DAPI) labeled (blue).

Supplementary Figure S3. Artificial heparinase-mediated heparan sulfate (HS) degradation is associated with local complement activation on glomerular endothelial cells (GECs). C3b deposits on GECs treated with medium only (0) or with increasing concentrations of heparinases I + II + III (1X: 0.2 mU/10^6 cells; 5X: 1 mU/10^6 cells; or 10X: 2 mU/10^6 cells) studied by immunofluorescence (IF) (green, ×20). Cell nuclei are 4′,6-diamidino-2-phenylindole (DAPI) labeled (blue).

Supplementary Figure S4. Transcriptional profile after overnight heme exposure of enzymes implicated in heparan sulfate (HS) biosynthesis. (**A,B**) Genic expression of enzymes in basal condition (0, white) or after 16 hours' exposure to 50 μmol/L of heme (h50, gray) on (**A**) human umbilical vein endothelial cells (HUVECs) and (**B**) glomerular endothelial cells (GECs). Results are presented as a ratio of the expression of the *HPRT* housekeeping gene (HUVECs: n = 3; GECs: n = 4).

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