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Original Article

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Adult and paediatric patients with minimal change nephrotic syndrome show no major alterations in glomerular expression of sulphated heparan sulphate domains

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Abstract

Background. Minimal change nephrotic syndrome (MCNS) is the most frequent form of nephrotic syndrome in childhood. In the glomerular basement membrane (GBM) of adult patients with MCNS, a reduced expression of a specific heparan sulphate (HS) domain has been reported. In children with MCNS, urinary activity of the HS-degrading enzyme heparanase was increased. It is, therefore, possible that a decreased GBM HS expression is associated with the pathogenesis of proteinuria in patients with MCNS.

Methods. In this study, HS in glomeruli of five adult and six paediatric patients with MCNS were analysed by immunofluorescence staining using four different antibodies, each defining a specific sulphated HS domain. The pediatric patients were subdivided into three groups depending on the presence or absence of podocyte foot process effacement, the level of proteinuria and prednisone administration at the time of the biopsy. In addition, kidneys of rats with adriamycin nephropathy (ADRN), a model for MCNS, were included in the study.

Results. Expression of sulphated HS domains was not aberrant in adult or paediatric patients compared with control subjects. Children with and without proteinuria had the same HS content. In contrast, rats with ADRN showed a decreased glomerular expression of sulphated HS domains.

Conclusions. These results suggest that in patients with MCNS proteinuria is not associated with major changes in glomerular expression of sulphated HS domains.

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Introduction

Minimal change nephrotic syndrome (MCNS), which is characterized by podocyte abnormalities and proteinuria [1,2], is the most frequent cause of nephrotic syndrome in childhood. This disease is proposed to be a disorder of T-cell dysfunction and the increased glomerular permeability may be due to a factor secreted from activated T-cells. The pathogenic cytokine, however, has not yet been identified.

It has been reported that loss of albumin into the urine is largely a result of loss of charge-selective permselectivity [3,4]. A reduction of fixed negative charges in the glomerular capillary wall has been noted [3,5]. The glycosaminoglycan heparan sulphate (HS) in the glomerular basement membrane (GBM) is assumed to be a major charge-selective component of the filtration barrier. The long, unbranched HS chains are composed of alternating glucosamine and D-glucuronic/L-iduronic acid residues, which are negatively charged due to the presence of multiple carboxylic groups and N–, 2–O–, 6–O–, and 3–O–sulphate groups [6]. In adult patients with MCNS, loss of HS in the GBM has been reported, whereas expression of the core protein of agrin, the major heparin sulfate protoglycans (HSPG) in the GBM, was not altered [7]. In children with steroid-sensitive nephrotic syndrome, urinary activity of heparanase, the endo-β-D-glucuronidase that catalyses the hydrolytic cleavage of HS, was found to be increased during relapse compared with patients with MCNS in

remission and control subjects. Urinary activity of heparanase was decreased in adult patients in remission and relapse compared with control subjects. The mRNA expression of heparanase by peripheral blood mononuclear cells was unaltered [8]. Another study showed that in co-culture with human glomerular epithelial cells (podocytes), peripheral blood mononuclear cells from patients with MCNS reduced the synthesis of secreted and cellular GAGs by the podocytes [9]. Taken together, these results indicate that an aberrant expression of HS in the GBM of patients with MCNS may contribute to the development of proteinuria. Therefore, in the present study, we investigated whether the pathogenesis of proteinuria in adult as well as paediatric patients with MCNS is associated with a decrease in glomerular HS expression using five different antibodies, defining specific HS domain structures.

Subjects and methods

Subjects

Kidney biopsies of five adult and six paediatric patients with MCNS were obtained from the Division of Nephrology and the Department of Pediatric Nephrology (Radboud University Nijmegen Medical Centre, Nijmegen, The Netherlands), respectively. The biopsies were taken after informed consent from all subjects.

Characteristics of the adult patients are summarized in Table 1. At the time of the biopsy, all patients had foot process effacement and proteinuria. None of the patients had received prednisone before the biopsy. Control renal tissue was obtained from either donor kidneys, which were not suitable for transplantation for anatomical reasons ($n = 3$), or from histologically normal parts of a kidney removed for renal adenocarcinoma ($n = 1$).

Table 2 summarizes the clinical characteristics of the paediatric patients. All patients were initially treated with prednisone. Frequently relapsing (i.e. two or more relapses within the first 6 months after the first attack, or four or more relapses in any 12-month period) as well as steroid-dependent patients [i.e. two consecutive relapses, or two of four relapses in any 6-month period, when a (usually reduced) dose of steroid is still given, or within 2 weeks of stopping steroid treatment] were included in the study. Three patient groups ($n = 2$ per group) were formed depending on the presence or absence of podocyte foot process effacement [visualized by electron microscopy (see subsequently)], the level of proteinuria, and prednisone administration at the day of the biopsy (Table 2). The following three groups were studied: (1) MCNS in remission, which is characterized by absence of foot process effacement, no proteinuria, but prednisone administration; (2) MCNS in relapse, which is characterized by foot process effacement, proteinuria, but no prednisone administration and (3) MCNS in relapse with steroid treatment, which is characterized by foot process effacement, proteinuria and prednisone administration. The biopsies in the patients

Table 1. Clinical characteristics of the adult patients with minimal change nephrotic syndrome

Subject	Sex	Age at biopsy (years)	Podocyte foot process effacement	Selectivity index at time of biopsy	Proteinuria at time of biopsy (g/day)	Prednisone dose at time of biopsy
1	♀	51	+	0.05	4.4	−
2	♀	28	+	0.19	9.1	−
3	♀	75	+	0.11	6.9	−
4	♂	77	+	<0.20	5–10	−
5	♂	71	+	>0.20	15.0	−

Selectivity index = clearance of IgG/clearance of albumin. Selectivity index < 0.10: very selective proteinuria; selectivity index > 0.20: a-selective proteinuria.
+, present; −, not present.

Table 2. Clinical characteristics of the paediatric patients with minimal change nephrotic syndrome

Subject	Sex	Age at biopsy	Indication biopsy	Podocyte foot process effacement	Proteinuria at time of biopsy (g/l)	Prednisone dose at time of biopsy
MCNS in remission						
1	♂	6 y 10 m	FR	−	−	15 mg/2 days
2	♀	3 y 5 m	FR	−	−	15 mg/day
MCNS in relapse						
1	♂	5 y 0 m	FR	+	1.5	−
2	♂	2 y 8 m	SD	+	2.1	−
MCNS in relapse + prednisone						
1	♂	4 y 0 m	FR	+	7.0	30 mg/day
2	♀	5 y 11 m	SD	+	16.5	60 mg/day

MCNS, minimal change nephrotic syndrome; y, years; m, months; FR, frequently relapsing; SD, steroid-dependent;
+, present; −, not present.

in remission, which were frequently relapsing, were performed as preparations for cyclophosphamide therapy. Renal tissue obtained from a boy biopsied because of postrenal anuria at the age of 3 years and 11 months was used as an age-matched control. This subject had no nephrotic syndrome, no proteinuria, no foot process effacement and the anuria disappeared rapidly after correcting the postrenal obstruction.

The human ethics committee of the Radboud University Nijmegen Medical Centre approved the study.

Electron microscopy

Kidney tissue was fixed in 2.5% (v/v) glutaraldehyde in 0.1 M Na-cacodylate buffer (pH 7.4) overnight at 4°C. Tissue was washed with 0.1 M Na-cacodylate buffer and postfixed in 2% (w/v) osmiumtetroxide (Electron Microscopy Sciences, Washington, PA, USA) in Palade buffer for 1 h. After washing with Palade buffer, tissue was dehydrated in ascending concentrations of ethanol, and embedded in Epon (Merck, Darmstadt, Germany) following Luft's procedure. Ultrathin sections were prepared, which were post-stained with 4% (w/v) uranyl acetate for 45 min and subsequently with lead citrate for 5 min. Sections were examined using a Jeol 1200 EX2 electron microscope.

Antibodies

Phage display-derived anti-HS antibodies were obtained by biopanning against HS or heparin, using bovine kidney HS (antibodies HS4C3 and HS3A8 or porcine intestinal mucosa heparin (antibodies EW3D10 and EW4G2). Production of single chain variable fragment anti-HS antibodies was performed as described [10]. Characteristics of the antibodies are depicted in Table 3.

Immunofluorescence staining

Human kidney biopsies were sliced to 2 µm cryosections and stored at −80°C until use. After air-drying, non-specific binding sites were blocked with PBS containing 2% (w/v) bovine serum albumin and 0.05% (v/v) Tween-20 (blocking buffer) for 20 min. Cryosections were then incubated with phage display-derived anti-HS antibodies in blocking buffer for 1 h. All antibodies were first titered on normal human kidney cryosections and used at the dilution that gave maximal specific fluorescence and minimal background staining. As a negative control, antibody MPB49 was used. This antibody is >95% identical to the other antibodies, but it is not reactive with HS and does not stain kidney tissue sections. Bound antibodies were detected by incubation with mouse IgG anti-VSV tag antibody P5D4 (1:10; Boehringer Mannheim, Mannheim, Germany), followed by Alexa 488-conjugated goat anti-mouse IgG (1:200; Molecular Probes, Eugene, OR), both for 45 min. After each antibody incubation, cryosections were washed three times for 5 min with PBS containing 0.1% (v/v) Tween-20. Finally, cryosections were fixed in 100% (v/v) ethanol for ∼10 s, air-dried and embedded in 10% (w/v) mowiol (Calbiochem, La Jolla, CA).

Glomerular staining intensity was examined using a Zeiss Axioscope microscope and scored semiquantitatively independenly by two observers on a scale of 0–10 [0 = no staining, 5 = 50% staining, 10 = 100% (maximum) staining]. The number of glomeruli counted in adult renal tissue was as follows: controls: $n = 20$; patient 1: $n = 4$; patient 2: $n = 6$; patient 3: $n = 6$; patient 4: $n = 2$; patient 5: $n = 4$. The number of glomeruli counted in paediatric biopsies was as follows: control: $n = 9$; MCNS in remission A: $n = 15$, B: $n = 16$; MCNS in relapse A: $n = 7$, B: $n = 9$; MCNS in relapse + prednisone A: $n = 4$, B: $n = 8$. Figures show the mean of the scores of the two observers. Each observer gave a mean score of all glomeruli per biopsy. Scoring between the two observers was consistent as deduced from regression analysis ($r^2 = 0.80$–0.90).

Adriamycin nephropathy animal model

Kidneys of male Wistar rats with adriamycin nephropathy (ADRN) were included in the study. In this animal model proteinuria and foot process effacement were observed. Unilateral ADRN was induced by clipping the left renal artery and vein through a midline abdominal incision, followed by injection via the tail vein with 1.5 mg/kg body weight of adriamycin. After 12 min, when adriamycin was cleared from the circulation, the clamp was removed. Rats were sacrificed after 6 and 12 weeks. Staining of the right kidney, which was exposed to adriamycin, was compared with that of the left kidney (control).

Statistical analysis

Differences between groups were determined by the non-parametric Mann-Whitney U-test using GraphPad

Table 3. Characteristics of domain-specific anti-heparan sulphate antibodies

Antibody	V_H CDR3 sequence	GAG used for selection	Essential chemical groups
HS4C3	GRRLKD	bovine kidney HS	NS, 6OS, 3OS
HS3A8	GMRPRL	bovine kidney HS	IdoA, NS, 2OS, 6OS (likely)
EW3D10	GRTVGRN	porcine intestinal mucosa heparin	S required, position(s) unknown[a]
EW4G2	GKVKLPN	porcine intestinal mucosa heparin	S required, position(s) unknown[a]
MPB49	WRNDRQ	–	–

Given are the antibody name, amino acid sequence of the V_H complementary determining region 3 (CDR3), the GAG used for selection and the preferred binding residues.
[a]Although the chemical nature of the specific HS/heparin structure recognized is not exactly known, the antibody defines a unique sulphated HS structure as demonstrated by its specific staining pattern on rat renal cryosections.
HS(PG), heparan sulphate (proteoglycan); IdoA, iduronic acid; S, sulphate; NS, N-sulphate; 2OS, 2-O-sulphate; 6OS, 6-O-sulphate, 3OS: 3-O-sulphate.

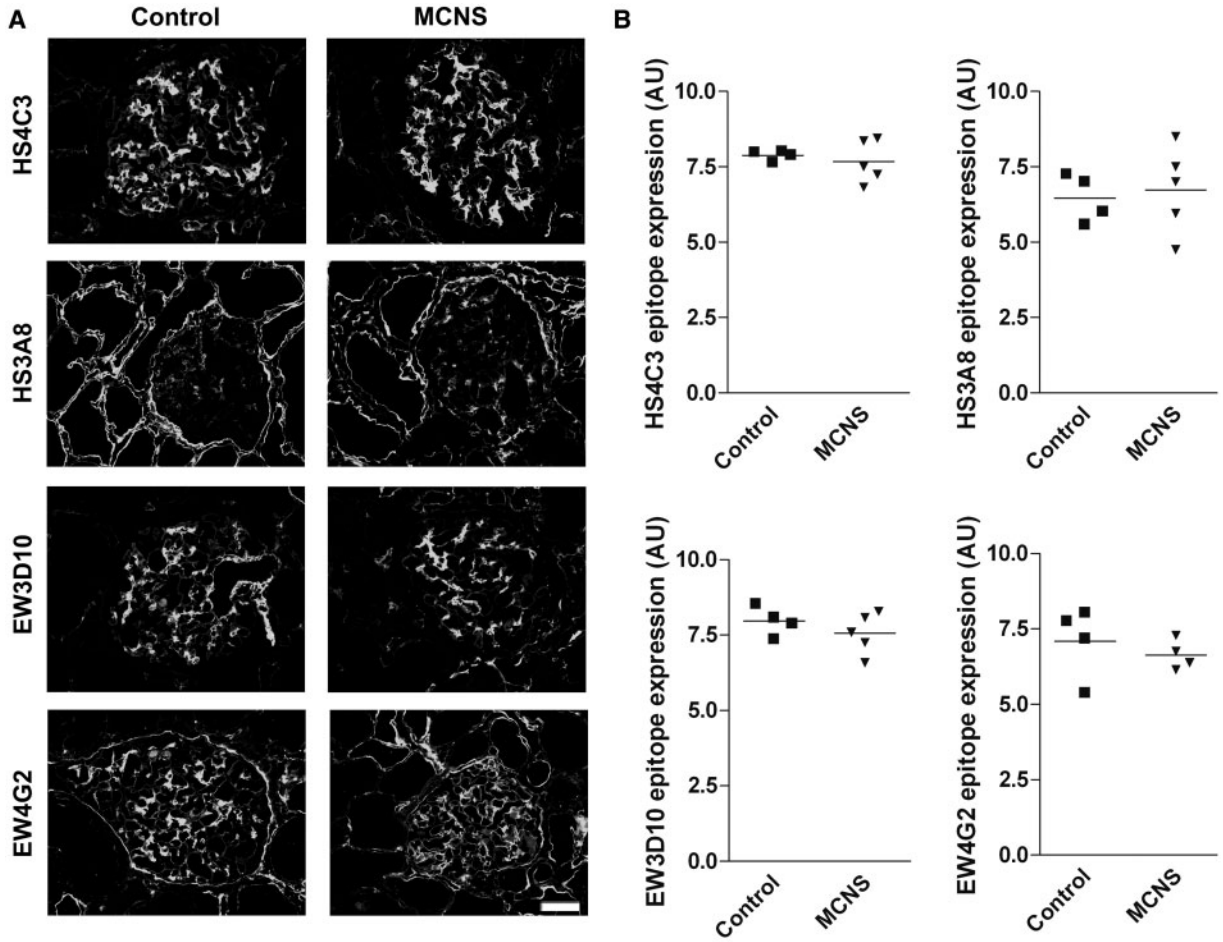

Fig. 1. Expression of sulphated HS domains in kidneys of adult control subjects and MCNS patients. (**A**) Immunofluorescence staining of renal tissue with anti-HS antibodies HS4C3, HS3A8, EW3D10 and EW4G2. Bar: 50 μm; magnification is identical for each photograph. (**B**) Quantification of immunofluorescence staining in arbitrary units (AU). The mean score of all glomeruli per biopsy is given. Note that glomerular HS staining is not aberrant in the patients.

Prism 4.0 (GraphPad Software, Inc., San Diego, CA, USA). Statistical significance was regarded if $P < 0.05$. Statistical analysis for the paediatric MCNS patients was not possible due to the small numbers of patients/group.

Results

The expression of HS in the GBM and mesangium of adult patients with MCNS was evaluated by immunofluorescence staining using a number of phage display-derived anti-HS antibodies, each defining a different sulphated HS domain. In control subjects, antibodies HS4C3, HS3A8, EW3D10 and EW4G2 showed a strong staining predominantly of the mesangial matrix, but also along the GBM. Expression was unaltered in glomeruli of patients with MCNS (Figure 1).

In addition to adult patients, paediatric patients with MCNS were included in the study. Different groups were made, based on the presence or absence of podocyte foot process effacement, level of proteinuria and prednisone administration. The MCNS patients in remission showed a normal renal ultrastructure. The three layers of the glomerular capillary wall formed by the fenestrated endothelial cells, the GBM and the podocyte foot processes, were clearly visible by routine electron microscopy. In contrast, the renal biopsies of the patients with MCNS in relapse showed obvious foot process effacement (Figure 2). Immunofluorescence staining with the phage display-derived anti-HS antibodies revealed no consistent differences in staining intensity between the MCNS patients and the control subject (Figure 3). In proteinuric patients with or without prednisone (MCNS in relapse + prednisone and MCNS in relapse, respectively) the profile of sulphated HS was also not different from the patients without proteinuria (MCNS in remission).

Taken together, no major differences in glomerular staining for HS could be detected by the different antibodies used. The staining procedure with the phage display-derived antibodies used was appropriate as we found a significantly reduced staining of HS

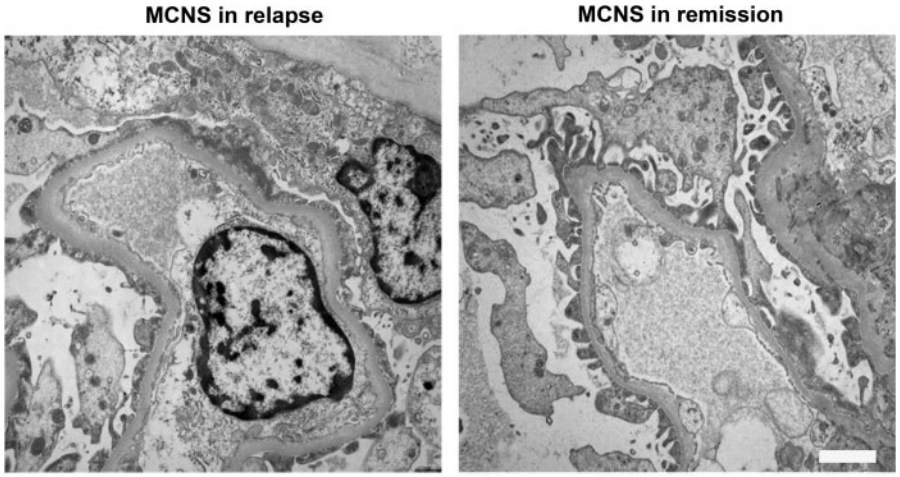

Fig. 2. Ultrastructure of glomeruli from paediatric patients with minimal change nephrotic syndrome in relapse and in remission. Note the podocyte foot process effacement in a patient in relapse, but not in a patient in remission. Bar: 2 μm; magnification is identical for each photograph.

with antibody HS4C3 in glomeruli of rats with ADRN (Figure 4).

Discussion

In adult patients with MCNS, loss of HS, but not of the core protein of agrin, which is the major HSPG in the GBM, has been reported [7]. In this study, we further investigated whether glomerular HS expression is aberrant in adult and paediatric patients with MCNS using immunofluorescence staining. The strength of our study is that we used four different anti-HS antibodies, each defining a specific sulphated HS domain, and analysed HS expression in paediatric patients with MCNS in different clinical conditions. No distinctions between different groups were made in the study of van den Born *et al.* [7] on HS in MCNS. With the tools used here, no significant differences in glomerular expression of sulphated HS domains could be detected in adult patients. Also, no major decrease was observed for the paediatric patients irrespective of the presence or absence of podocyte foot process effacement, level of proteinuria and prednisone administration. In paediatric patients with steroid-sensitive nephrotic syndrome, urinary activity of heparanase was increased during relapse, but not during remission [8]. However, amounts of HS excreted into the urine were not aberrant [11], which supports our observation that no HS has been removed from the glomeruli of paediatric patients with MCNS.

It has been noticed that proteinuria in MCNS is largely a result of loss of charge-selective permselectivity [3,4]. HS in the GBM has been assumed to play an important role in preventing urinary loss of albumin by providing fixed negative charges to the glomerular capillary wall. However, recent data indicate that HS does not play a direct major role in the charge-selective barrier properties of the GBM. Removal of HS from the GBM by injection of Wistar rats with the bacterial HS-degrading enzyme heparinase III did not result in proteinuria [12]. In podocyte-specific agrin (major HSPG in the GBM) knock-out mice, no proteinuria was observed [13], which was also the case for mice lacking the HS-bearing, exon 3-coded, part of perlecan (minor HSPG in the GBM) [14]. Transgenic mice overexpressing human heparanase only had slightly elevated levels of proteinuria [15]. In addition, it should be noted that no aberrant glomerular HS expression was detected in proteinuric patients with congenital nephrotic syndrome of the Finnish type [16] or IgA nephropathy [7]. Taken together, these observations indicate that other mechanisms than a decrease in glomerular HS expression probably contribute to the development of proteinuria in patients with MCNS.

If proteinuria in MCNS is not the result of loss of sulphated HS, what may then be its cause? The observations of podocyte foot process effacement [1,2,17], a decrease in the number of slit diaphragms [2], and possible decreased expression and/or redistribution of the slit diaphragm-associated protein nephrin [1,17] and the podocytic protein synaptopodin [18] in MCNS suggest that the podocyte and/or slit diaphragm have a role in the defective function of the glomerular filtration barrier. In addition, α- and β-dystroglycans were reduced in relapsing MCNS, but returned to normal after steroid treatment [19], indicating that proteinuria in MCNS may also be the result of a decreased adhesion of podocytes to the GBM.

If there is no loss of sulphated HS in the GBM of patients with MCNS, how then can the observed reduction of fixed negative charges in the glomerular capillary wall be explained? Experimental MCNS, induced by injection of rats with puromycin aminonucleoside, was characterized by the loss of negatively

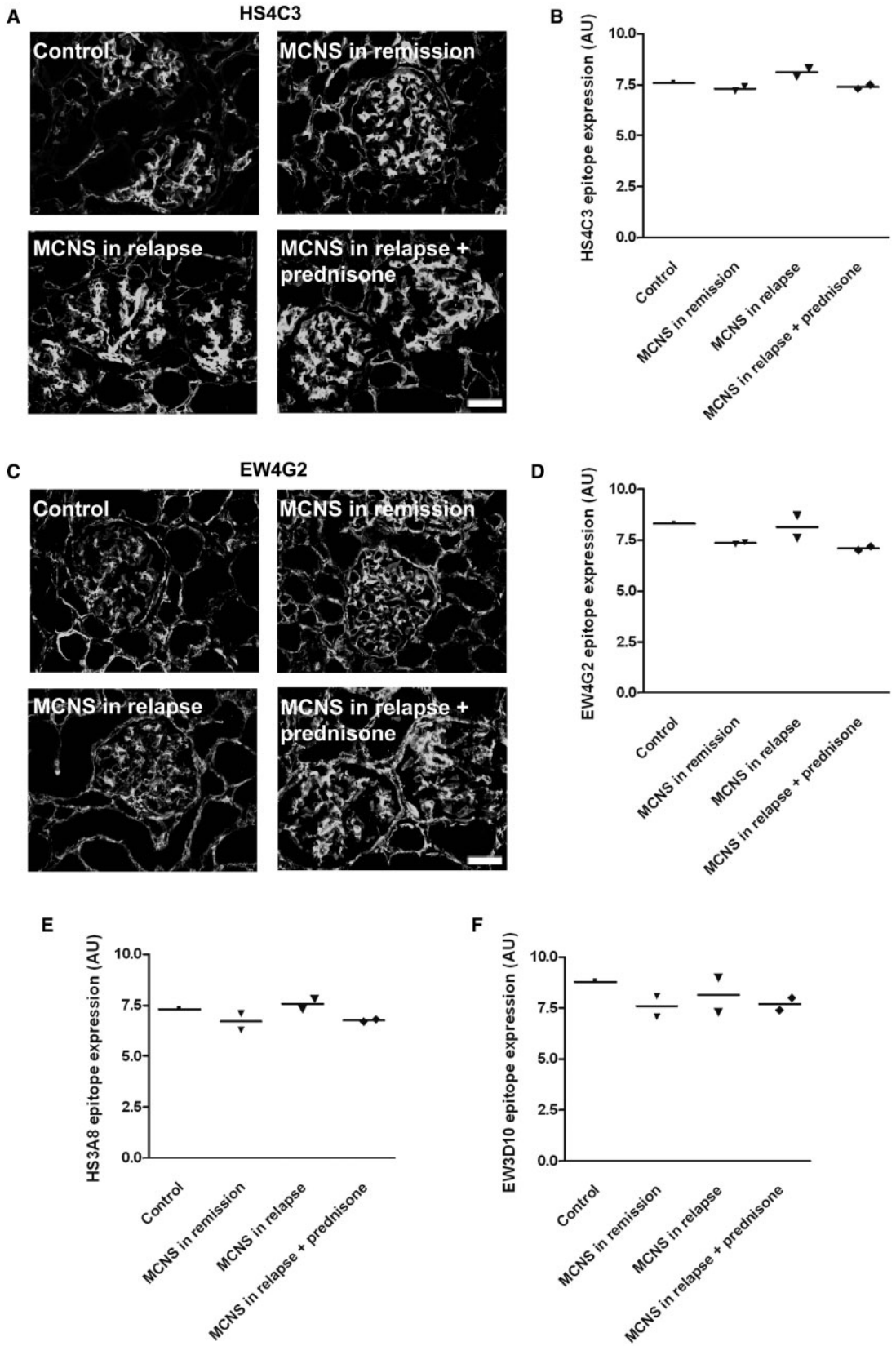

Fig. 3. Expression of sulphated HS domains in kidneys of a paediatric control subject and MCNS patients in remission, relapse and relapse with prednisone treatment. (**A**, **C**) Immunofluorescence staining of renal tissue with anti-HS antibodies HS4C3 (A) and EW4G2 (C). Bar: 50 μm; magnification is identical for each photograph. (**B**, **D**, **E**, **F**) Quantification of immunofluorescence staining with HS4C3 (B), EW4G2 (D), HS3A8 (E) and EW3D10 (E) in arbitrary units (AU). The mean score of all glomeruli per biopsy is given. Note that glomerular HS staining is not aberrant in any patient group.

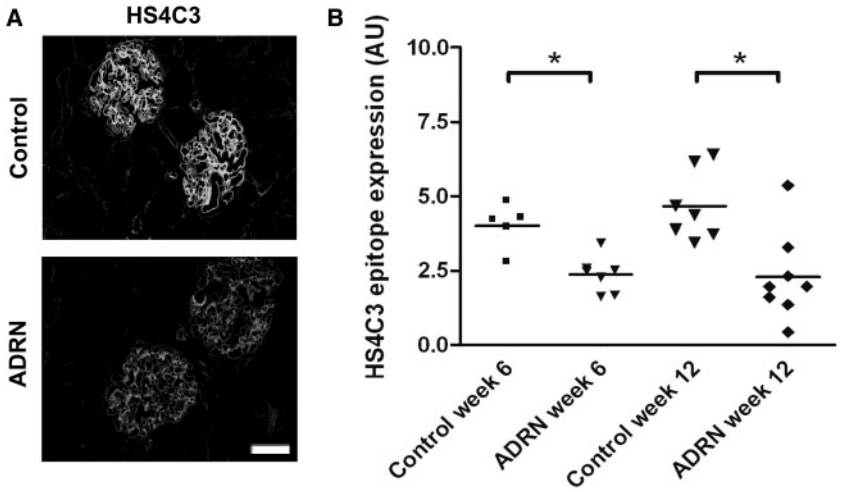

Fig. 4. Expression of sulphated HS domains, defined by antibody HS4C3, in kidneys of Wistar rats unilaterally exposed to adriamycin. (**A**) Immunofluorescence staining of rat renal tissue with anti-HS antibody HS4C3. The non-exposed (control) kidney was compared with the exposed (ADRN) kidney. Bar: 50 μm; magnification is identical for each photograph. (**B**) Quantification of immunofluorescence staining in arbitrary units (AU). The mean score of all glomeruli per kidney is given. Note the decrease in glomerular HS staining in ADRN rats. $^{*}P < 0.05$.

charged neuraminic acid residues, attached to the protein podocalyxin, which is predominantly expressed at the podocyte cell surface, but also in the endothelial glycocalyx [20, 21]. In paediatric MCNS patients with proteinuria, a very weak to absent glomerular reactivity of colloidal iron, reported to be specific for neuraminic acid, was detected [20]. In addition, removal of glomerular neuraminic acid after injection of mice and rats with the enzyme neuraminidase resulted in proteinuria [12]. Furthermore, recently, it was found in cell culture studies that removal of neuraminic acid from the endothelial glycocalyx increased the passage of albumin [23]. These results indicate that neuraminic acid in the endothelial glycocalyx and/or at the podocyte cell surface may be an important determinant for the charge-selective properties of the glomerular capillary wall, and be involved in the pathophysiology of MCNS.

To conclude, no major alterations in glomerular expression of sulphated HS domains were observed in paediatric patients with MCNS irrespective of the presence or absence of podocyte foot process effacement, the level of proteinuria and prednisone administration. In adult patients, sulphated HS domains were also not aberrant. These results indicate that proteinuria observed in adult as well as paediatric patients with MCNS may not be due to major alterations in glomerular expression of sulphated HS domains.

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Conflict of interest statement. None declared.

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