Expression of sialidase and dystroglycan in human glomerular diseases

Nils P. J. Vogtländer[1], Johan van der Vlag[1], Marinka A. H. Bakker[1], Henry B. Dijkman[2],
Ron A. Wevers[3], Kevin P. Campbell[4], Jack F. M. Wetzels[1] and Jo H. M. Berden[1]

[1]Department of Nephrology, Nephrology Research Laboratory, Nijmegen Centre for Molecular Life Sciences, [2]Department of
Pathology, [3]Department of Laboratory Medicine, Laboratory of Pediatrics and Neurology, Radboud University Nijmegen Medical
Centre, Nijmegen, The Netherlands and [4]Department of Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City,
IA, USA

Correspondence and offprint requests to: J. H. M. Berden; E-mail: j.berden@nier.umcn.nl

Abstract

Background. α-Dystroglycan (α-DG) is a negatively
charged glycoprotein that covers the surface of podocytes.
A decreased glomerular expression of α-DG has been de-
scribed in minimal change nephropathy (MCN), but not in
focal segmental glomerulosclerosis (FSGS). This was sug-
gested as a tool to distinguish these diseases. Sialic acid
is a negatively charged carbohydrate extensively present
on both α-DG and podocalyxin, which is also expressed
on podocytes. Intrarenal perfusion with bacterial sialidase
leads to foot process effacement and proteinuria. This is
the first study on the expression of endogenous glomerular
sialidase; furthermore, the expression of dystroglycan was
re-evaluated.
Methods. The expression of α-DG and sialidase was in-
vestigated by immunofluorescence in kidney biopsies of
patients with MCN ($n = 5$), FSGS ($n = 15$), prolifera-
tive lupus nephritis (LN, $n = 9$), membranous glomeru-
lopathy (MG, $n = 10$) and normal human kidneys (NHK,
$n = 4$). The urinary sialic acid concentration was measured
using a newly developed LC-tandem mass spectrometry
method.
Results. A 3-fold increased glomerular expression of sial-
idase was found in MG, accompanied with an increased
urinary sialic acid concentration in two MG patients. How-
ever, we did not observe major changes in the expression
of α-DG in patients with the above-mentioned glomerular
diseases compared to NHK, also not between MCN and
FSGS.
Conclusions. Endogenous glomerular sialidase expression
is increased in MG, which might represent a novel mech-
anism for the loss of negative charge in the glomerular
capillary filter. The expression of dystroglycan cannot be
used as a diagnostic tool to differentiate between glomerular
diseases.

Keywords: dystroglycan; podocalyxin; podocyte; sialic acid; sialidase

Introduction

The glomerular capillary filter consists of the fenes-
trated endothelial cells, the glomerular basement membrane
(GBM) and the visceral epithelial cells or podocytes. It al-
lows size- and charge-dependent filtration, which is dis-
turbed in proteinuric diseases. Minimal change nephropa-
thy (MCN) is the most common cause of the nephrotic
syndrome in children and accounts for 10–15% of cases of
the nephrotic syndrome in adults [1]. In adult patients, the
most common causes of the idiopathic nephrotic syndrome
are focal segmental glomerulosclerosis (FSGS) and mem-
branous glomerulopathy (MG). The kidney is also often
involved in systemic diseases. The best-known example is
systemic lupus nephritis (LN), which has a variable histol-
ogy, which may include a membranous pattern of injury
and/or mesangial, mesangiocapillary or extracapillary pro-
liferation. In these glomerular diseases, the negative charge
is diminished in the glomerular capillary filter, both in-
cluding the basement membrane [2] and the surface of
podocytes [3,4]. This loss of negative charge has mainly
been attributed to heparan sulfate, but may also involve
sialic acid residues [5,6]. In recent years, the importance of
the anionic sites within the GBM for the charge-dependent
permeabillity has been challenged [7,8]. The removal of
heparan sulfate from the GBM by different approaches did
not lead to proteinuria [9–11]. In the past, tracer studies
have indicated that the glomerular capillary barrier acts as
a charge selective filter. If this charge-dependent filtration
does not occur in the GBM, where does it reside? Two possi-
bilities have been proposed: the endothelial glycocalyx [12]
and the podocyte including the slit diaphragm [3]. Since an-
ionic charge is decreased in many glomerular diseases, we
have focussed in this analysis on two components carrying
the negative charge on podocytes, namely the sialoprotein
dystroglycan and sialic acid itself.

Dystroglycan is a negatively charged glycoprotein that
covers the basolateral and apical cell membranes of the
podocyte [13]. It is encoded by the gene *DAG 1* and

Table 1. Antibodies, lectins and conjugates

Primary antibody or lectin	Epitope	Dilution primary antibody	Conjugates	Dilution secondary antibody
JM72[a]	Core protein agrin (central)	1:400	Goat anti-mouse IgG1 Alexa fluor 568[b]	1:200
MI 90[a]	Core protein agrin (N-terminal)	1:400	Goat anti-Syrian hamster IgG CY3[c]	1:200
IIH6[d]	α-Dystroglycan carbohydrates	1:20	Goat anti-mouse IgG Alexa fluor 488[b]	1:200
VIA4.1[d]	α-Dystroglycan carbohydrates	1:5	Goat anti-mouse IgG Alexa fluor 488[b]	1:200
NCL-BDG[e]	β-Dystroglycan	1:10	Goat anti-mouse IgG Alexa fluor 488[b]	1:200
ShαRbDG 549–572[a]	α-Dystroglycan core protein aa 549–572	1:25	Goat anti-sheep IgG Alexa fluor 488[b]	1:1000
Sialidase[f]	Neu1	1:200	Goat anti-rabbit Alexa fluor568[b]	1:200
Vicia villosa lectin[g] (VVL)	Beta-linked or Ser/Thr-linked term GalNAc	1:1000	Streptavidin Alexa fluor 488[b]	1:200
Peanut agglutinin[g] (PNA)	Galβ1–3GalNAc	1:1000	Streptavidin Alexa fluor 488[b]	1:200
Sambuccus nigra agglutinin (SNA)	Sialic acid with pre-terminal α-2,6 galactose	1:1000	Streptavidin Alexa fluor 488[b]	1:200

Neu1, sialidase 1; Gal, galactose; GalNAc, *N*-acetylgalactosamine.
[a]Own laboratory [32].
[b]Molecular Probes, Leiden, the Netherlands.
[c]Jackson, Immunoresearch Laboratories, Inc., Westgrove, Pennsylvania.
[d]Kevin Campbell.
[e]Monosan (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK).
[f]Alexey Pshezhetsky, Université de Montreal, Service de Génétique Médicale, Département de Pédiatrie, Hôpital Sainte-Justine, Montréal, Québec, Canada [39].
[g]Vector Laboratories Inc., Burlingame, Canada.

post-translationally spliced into α and β subunits [14]. The β subunit (β-DG) is a transmembrane protein that binds to the extracellular α-dystroglycan (α-DG) and in podocytes to the intracellular utrophin [15,16]. At the basolateral cell membrane, α-DG binds laminin G modules in the GBM, present in laminin, agrin and perlecan [17]. At the apical cell membrane, α-DG is involved in signalling events [18] and has a putative function in the maintenance of the patency of the filtration slit, in analogy to what has been demonstrated for podocalyxin [13,19]. α-DG undergoes heavy glycosylation that includes terminal negatively charged sialic acid residues. The expression of a glyco-epitope on α-DG is decreased in adriamycin nephropathy and IL-13 overexpressing rats [16,17,20] both resembling MCN, and indeed, it has been reported that this glyco-epitope on α-DG is selectively decreased in MCN but not in FSGS [21]. On the other hand, no significant changes have been reported in puromycin aminonucleoside nephrosis or passive Heymann nephritis [16,22].

The negative charge of the sialic acid residue that covers the apical podocyte is important for the patency of the filtration slit, as *in vivo* removal of the negatively charged sialic acids on podocalyxin and dystroglycan by bacterial sialidase results in proteinuria and foot process effacement [5,6,19,23]. Recently, sialic acid in the glycocalyx on glomerular endothelial cells was also assigned an important role in the charge selective permeability of the glomerular capillary filter, as *in vitro* treatment with sialidase resulted in an increased permeability to albumin [12].

The crucial function of sialoproteins on podocytes was further illustrated by podocyte foot process effacement, GBM splitting and severe proteinuria and haematuria in mice carrying a mutated form of uridine diphospho-*N*-acetylglucosamine-2-epimerase/*N*-acetylmannosamine kinase, the key enzyme in the assembly of sialic acid. Remarkably, the lesions in these knock-in mice can be partially corrected by dietary addition of *N*-acetylmannosamine

[24], a finding that puts sialic acid in an exciting new perspective [25]. Sialidases or neuraminidases are a family of endoglycosidases that catalyse the hydrolytic cleavage of non-reducing sialic acid residues linked to (mono- or) oligosaccharide chains of glycoproteins.

We hypothesize that endogenous sialidase might play a role in the cleavage of terminal sialic acids from both podocalyxin and α-DG on the podocyte in glomerular disease. Sialidase expression has, to our knowledge, never been studied in glomerular diseases by immunohistological methods. However, limited experimental data suggest increased sialidase activity in glomeruli from streptozotocin-induced diabetic rats and in adriamycin nephropathy [26], but unchanged sialidase activity in puromycin aminonucleoside-treated rats [27–29].

In the present study, we therefore evaluated the glomerular expression of dystroglycan, sialic acid and sialidase in human glomerular diseases.

Patients and methods

Biopsy and urine collection

We selected renal biopsy tissue of adult patients with proteinuria >0.5 g/day, a diagnosis of FSGS, MCN, MG or LN (ISN/RPS class III or IV). Four cadaveric kidneys, not suitable for transplantation because of anatomical reasons, served as control [normal human kidney (NHK)]. Clinical data were retrieved from the patients' records. The selectivity index of proteinuria was calculated as the clearance of IgG divided by the clearance of transferrin [30]. Biopsies and 2-μm sections were stored at −80°C. Urine was collected during 2 h at the time of biopsy, as previously described [31].

Immunofluorescence

Immunofluorescence was performed as previously described [13]. Antibodies, lectins and conjugates were used in the concentrations listed in Table 1. Agrin in the GBM was probed with the monoclonal antibody MoAb JM72 or MI 90 [32], in order to provide insight into the exact localization of the other dye in the double staining and to identify sclerotic glomerular lesions, which were not scored. The expression of the agrin

Table 2. Patient characteristics

	MCN	FSGS	MG	LN
N	5	15	10	9
Male (N)	2	9	6	0
Age[a] (years)	70.7 (27–77)	54.7 (19–69)	56.1 (32–72)	25.6 (18–74)
Stage/class I–II–III–IV (N)			3-4-3-0	0-0-5-4
Endogenous creatinine clearance[a] (ml/min)	78 (25–85)	109 (24–165)	105 (30–155)	91 (35–137)
Serum albumin[a] (g/l)	16 (15–34)	24 (14–44)	22 (10–24)	32 (23–45)
Proteinuria[a] (g/day)	7.8 (3.4–19)	9.0 (4–15)	8.2 (2.5–16)	2.7 (0.6–15)
Selectivity index[a]	0.09 (0.05–0.27)	0.22 (0.07–0.40)	0.20 (0.08–0.27)	na

[a]Values are given as median and range.
MCN, minimal change nephropathy; FSGS, focal segmental glomerulosclerosis; MG, membranous glomerulopathy; LN, lupus nephritis; na, not available.

Table 3. Scores of markers in glomerular disease

Antibody	NHK	MCN	FSGS	MG	LN	Interclass	ANOVA P-value
α-DG (ShαRbDG)	1.0 ± 0.2	0.6 ± 0.2	0.6 ± 0.1	0.7 ± 0.9	0.7 ± 0.3	0.37	0.019
β-DG (NCL-bDG)	3.3 ± 0.2	3.4 ± 0.3	3.2 ± 0.5	3.0 ± 0.4	3.0 ± 0.4	0.83	
α-DG (VIA4.1)	2.9 ± 0.6	2.8 ± 0.5	2.9 ± 0.4	3.1 ± 0.3	2.2 ± 0.9	0.86	0.032
α-DG (IIH6)	3.4 ± 0.4	2.8 ± 0.2	2.9 ± 0.5	2.9 ± 0.8	2.8 ± 0.7	0.63	
Sialidase	0.9 ± 0.2	0.9 ± 0.4	1.1 ± 0.6	3.2 ± 0.4	1.0 ± 1.0	0.93	0.000
VVL	1.5 ± 0.4	1.1 ± 0.5	1.7 ± 0.4	2.0 ± 0.6	2.2 ± 0.5	0.60	0.005
PNA	0.2 ± 0.3	0.4 ± 0.2	0.8 ± 0.8	0.3 ± 0.2	1.5 ± 1.0	0.85	0.016

The mean staining intensity of podocytes was scored per glomerulus on a linear scale of 0–4, by two of the investigators on blinded sections.
Antibodies and lectins: VIA4.1 and IIH6 are monoclonal antibodies directed at glycoepitopes on α-dystroglycan. ShαRbDG is a polyclonal antibody directed at the core protein of α-dystroglycan. NCLbDG is a monoclonal antibody directed at β-dystroglycan.
VVL and PNA bind to carbohydrate epitopes containing galactose (Gal) and/or *N*-acetylgalactosamine (GalNAc) residues, which each can be masked by terminal sialic acid residues.
MCN, minimal change nephropathy; FSGS, focal segmental glomerulosclerosis; MG, membranous glomerulopathy; LN, lupus nephritis.

core protein was similar amongst all biopsies, and therefore, it is a suitable marker for the exact localization of the capillary filter. For staining with lectins, the kidney sections were fixed during 10 min in acetone at 4°C. In one experiment, NHK sections were exposed to neuraminidase (sialidase) from *Vibrio cholerae* 0.04 U/ml (preferably α-2,6 linkage, Sigma) in 50 mM sodiumacetate and 1 mM calciumchloride pH 5.5 for 2 h at 37°C. The reaction was stopped by fixation in acetone 4°C for 10 min.

After drying in the air, endogenous biotin was blocked with 1 μg/ml streptavidin for 15 min (Sigma, St Louis, MO, USA). After washing with phosphate buffered saline (PBS, Merck, Darmstadt, Germany) the excess streptavidin was blocked with 0.1 μg/ml d-biotin for 15 min (Sigma) and washed in PBS. Thereafter, the glyco-epitopes were probed with the biotin-labelled lectins (Table 1) for 45 min, washed with PBS and finally visualized with streptavidin Alexa fluor for 45 min, all in PBS at room temperature. After washing with PBS, the kidney sections were embedded in Vectashield mounting medium H-1000 (Vector). Pictures were taken with a Leica DM 4000 B Discovery microscope and camera (Leica Microsystems, Wetzlar, Germany).

The mean staining intensity of podocytes was scored per glomerulus on a linear scale of 0–4, by two of the investigators on blinded sections.

Free urinary sialic acid measurement

Urinary free sialic acid (SA), normalized to the urinary creatinine concentration, was analysed by LC-tandem mass spectrometry with $^{13}C_3$-labelled neuraminic acid as internal standard ($^{13}C_3$SA Sigma Aldrich). Briefly, 5 μl urine diluted to 0.1 mM creatinine and spiked with internal standard is injected onto an Atlantis dC18 HPLC column (186001295, 2.1 × 100 mm, 3 μm; Waters, Etten-Leur, the Netherlands). Sialic acid is eluted at 0.25 ml/min with 1% methanol and 0.3% formic acid. After 1.6 min, the column is washed at 0.28 ml/min for 2.5 min with 50% methanol and 0.3% formic acid. Using negative electron spray tandem mass spectrometry, the transition of neuraminic acid to glycidic acid was monitored. The Quattro LC tandem MS (Waters) is operated in the negative electron spray ionization mode with a capillary voltage of 3 kV and a cone voltage of 22 V. Source and desolvation temperatures are set to 80°C and 300°C, respectively. Nitrogen is used as desolvation gas (630 L/H) and argon as collision gas (1.7×10^{-3} mbar). The collision energy was set at 20 eV. Free urinary sialic acid reference values for patients >10 years were 1–11 μmol/mmol creatinine.

Statistics

Patient's characteristics and expression of glomerular markers were analysed using SPSS 14.0.0 (SPSS Inc., Chicago, IL, USA). Interclass scores were calculated to quantify inter-observer variation, which was defined as good when the interclass score was 0.6–0.8 and excellent if ≥0.8 [33]. To compare means between all groups, the ANOVA was performed. To compare means of two groups, the student *t*-test was performed, two-tailed with Bonferroni correction. To compare means for skewed distributed data (sialic acid), the Mann–Whitney *U* test was performed. Correlations were calculated by using Pearson logistic regression.

Results

The patient characteristics are depicted in Table 2. The glomerular scores of the various markers are given in Table 3. No correlation of any staining was found with the age of the patient.

Glomerular expression of dystroglycan in proteinuric diseases

We used ShαRbDG as an antibody to visualize the expression of the core protein of α-DG. This antibody barely reacts with NHK [17], but epitopes could be revealed in glomerular diseases as it is directed at the binding site of
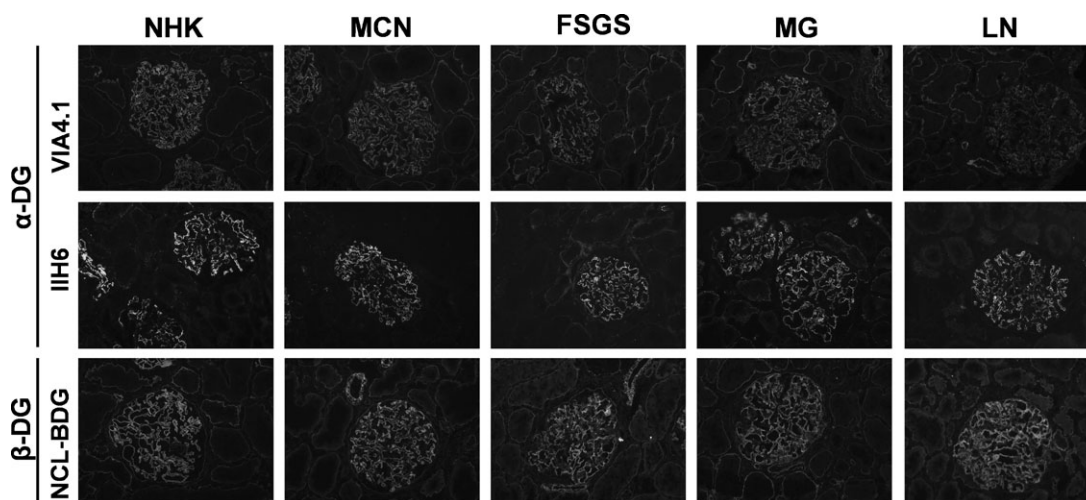
Fig. 1. Dystroglycan in glomerular disease. The expression of α-dystroglycan (α-DG) as studied by MoAb VIA 4.1 (upper panels) was only decreased in lupus nephritis (LN). The expression was similar when studied by MoAb IIH6 for all glomerular diseases (middle row panels). Both antibodies are directed at carbohydrate epitopes on α-dystroglycan. The expression of β-dystroglycan (β-DG) (NCL-bDG) was similar amongst the glomerular diseases and normal human kidney (lower panels). See Table 3 for the average scores and statistics.

α- to β-DG. However, the expression of the epitope in the α-DG core protein remained very low in all patient groups evaluated, resulting in a low interclass coefficient (0.37). Therefore, we used β-DG as a marker of the DG complex (see Figure 1 and Table 3). The expression of β-DG was similar in all groups and not different from NHK.

We next analysed the expression of the glyco-epitopes on α-DG using VIA4.1 and IIH6 (Figure 1 and Table 3). Except for LN, no major changes were noted. Specifically, there was no difference in the staining intensity of either antibody between MCN and FSGS.

FSGS is a description of a histological pattern of injury and not of a disease entity. FSGS can be primary (idiopathic) or secondary to hypertension or hyperfiltration. Therefore, we have reanalysed the data on α-DG staining after dividing cases into primary and secondary FSGS based on clinical criteria [34].

There were eight patients with primary FSGS. Their mean age was 39 years, creatinine clearance 121 ml/min, mean serum albumin 21 g/l, proteinuria 10.2 g/24 h and selectivity index 0.19. We identified seven patients with secondary FSGS, mean age 57 years, creatinine clearance 78 ml/min, mean serum albumin 28 g/l, and proteinuria 8.6 g/24 h and selectivity index 0.28. Again we observed no differences in the expression of the glyco-epitopes on α-DG on comparing these two FSGS groups. When comparing MCN and primary FSGS, staining intensity for VIA4.1 was 3.1 versus 2.8 and for IIH6 it was 2.9 versus 2.8, respectively.

Glomerular expression of sialidase in proteinuric diseases

One of the key findings from this study was a 3-fold increase in sialidase expression in patients with MG compared to other glomerular disease and NHK. This increase was most pronounced in the capillary filter, most likely podocytes, although the mesangial expression was also in-

creased (Figure 2). In NHK, sialidase was expressed at a low level in the mesangium and at the vascular pole of the glomerulus (Figure 2). The variation of the expression level of sialidase among MG patients was rather small (mean = 3.21; SD = 0.44), in contrast to proteinuria (8.7 g/day SD = 4.4 g/day). Therefore, we were unable to detect a correlation of sialidase expression with proteinuria ($P = 0.393$) in our patients with MG. Nevertheless, we would like to relate the increased sialidase expression in patients with MG to enzymatic activity. However, given the limited tissue in renal biopsies, it is not feasible to measure sialidase enzyme activity biochemically. Therefore, as an indirect measure for sialidase activity, we measured urinary sialic acid normalized to creatinine in urines as depicted in Figure 3. Urine samples of eight patients with MG collected at the time of biopsy were available, but only two urine samples of patients with MCN, three of patients with FSGS, and zero of patients with LN. The maximum normal reference value, as determined in a large validation cohort used for the development and implementation of the method, is depicted by the grey dotted line (11 μmol/mmol creatinine). The concentration of sialic acid was found to be increased above this threshold in the urine samples of two patients with MG ($N = 8$), but in none of patients with FSGS ($N = 3$) MCN ($N = 2$) or healthy volunteers ($N = 7$). The increase in urinary sialic acid concentration was significant in patients with MG in comparison to healthy volunteers ($P = 0.003$, Mann–Whitney U test); the ANOVA showed a strong trend towards significance ($P = 0.054$). Probing of renal sections with specific lectins could be another approach to show endogenous sialidase activity *in situ*. Other groups have shown *in vivo* and *in vitro* that specific desialization of sialoproteins unmasks *N*-acetylgalactosamine (GalNAc) and galactose (GAL) residues [35,36]. Indeed, treatment of NHK sections with sialidase revealed an enhancement of PNA staining and to a lesser extent of VVL staining (Figure 4). Both lectins recognize terminal Gal and GalNAc residues, which become accessible after the removal of
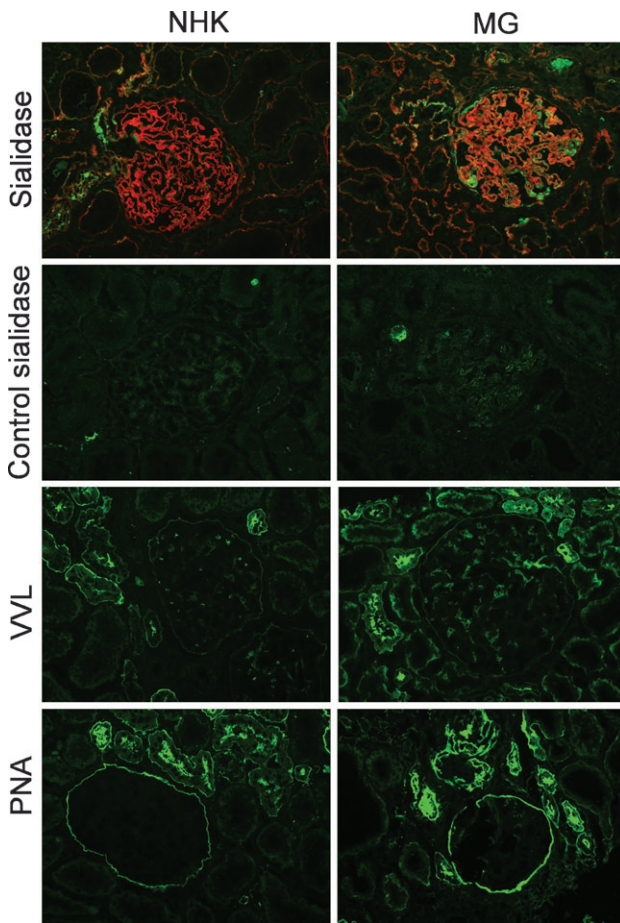
Fig. 2. Sialidase expression is increased in membranous glomerulopathy. Sialidase (green) is expressed at a low level in the mesangium and near the vascular pole of the glomeruli of NHK. A significant increase in sialidase expression was observed in patients with MG compared to other glomerular disease and NHK. This increase was most pronounced at the capillary filter as revealed by co-staining for the heparan sulfate proteoglycan core protein agrin (red). Non-immune rabbit serum served as control. See Table 3 for the average scores and statistics.

Fig. 3. Urinary sialic acid normalized for creatinine in patients and controls. Sialic acid and creatinine were measured in available urines of patients and controls. The maximum normal reference value, as determined in a large validation cohort during the development and implementation of the assay, is depicted by the grey dotted line (11 μmol/mmol creatinine). The concentration of sialic acid was increased above this threshold in the urine of two patients with MG ($N = 8$), but in none of patients with FSGS ($N = 3$), MCN ($N = 2$) or healthy volunteers ($N = 7$). The increase in urinary sialic acid concentration was significant in patients with MG in comparison to healthy volunteers (Mann–Whitney U); ANOVA showed a strong trend towards significance ($P = 0.054$).

terminal sialic acid [37]. Because we observed in biopsies of patients with MG an increased sialidase expression, we analysed whether the same changes in lectin staining could be observed as after treatment of normal kidney sections with sialidase. To our surprise, we did not find an enhancement of PNA staining in MG. The binding of VVL was slightly but significantly higher in patients with MG, but there was no correlation with the expression of sialidase. Table 3 also indicates that the binding of VVL and PNA was slightly increased in patients with LN, but this was not related to an increased sialidase expression.

Discussion

Sialidases or neuraminidases are a family of endoglycosidases that catalyse the hydrolytic cleavage of non-reducing sialic acid residues linked to (mono- or) oligosaccharide chains of glycoproteins. There are four subtype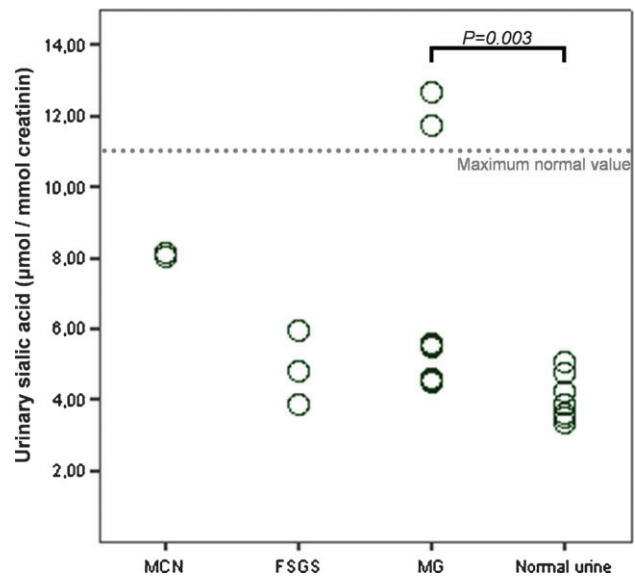s of mammalian sialidase, originally characterized by their localization: lysosomal neuraminidase 1 (NEU1); cytosolic neuramidase 2; plasma membrane-associated neuramidase 3 and mitochondrial neuramidase 4. In the kidney, up to now only the biochemical activity of sialidase has been studied, showing higher activity in tubuli than in glomeruli [29,38]. Sialidase activity in isolated homogenized glomeruli was increased in streptozotocin-induced diabetes in rats and correlated with albuminuria and transferrin excretion [27,28]. However, the activity of sialidase remained stable in puromycin aminonucleoside nephrosis in rats [29]. These few studies were hampered by the quick loss of sialidase activity at 4°C and at freeze thaw steps. To determine the expression and exact localization of sialidase, immunofluorescence forms a suitable method. We used an antibody for NEU1, which is specific and shows no cross reactivity with the other neuraminidases [39]. A low glomerular expression of sialidase was found in NHK, MCN, FSGS and LN. However, a significant increase in glomerular expression of sialidase was found in MG, predominantly in the podocyte. The upregulation of sialidase in MG but not in the other glomerular diseases indicates that proteinuria by itself is not sufficient to cause this upregulation. Moreover, the immune complex deposition by itself does not induce sialidase expression, as the sialidase expression in LN is not increased. The sialidase expression may be a consequence of specific podocyte damage occurring in MG. Previous experiments have shown that *in situ* treatment with bacterial sialidase unmasks the pre-terminal galactose-*N*-acetylgalactosamine after the removal of the terminal sialic acid [35,36]. If we treated NHK
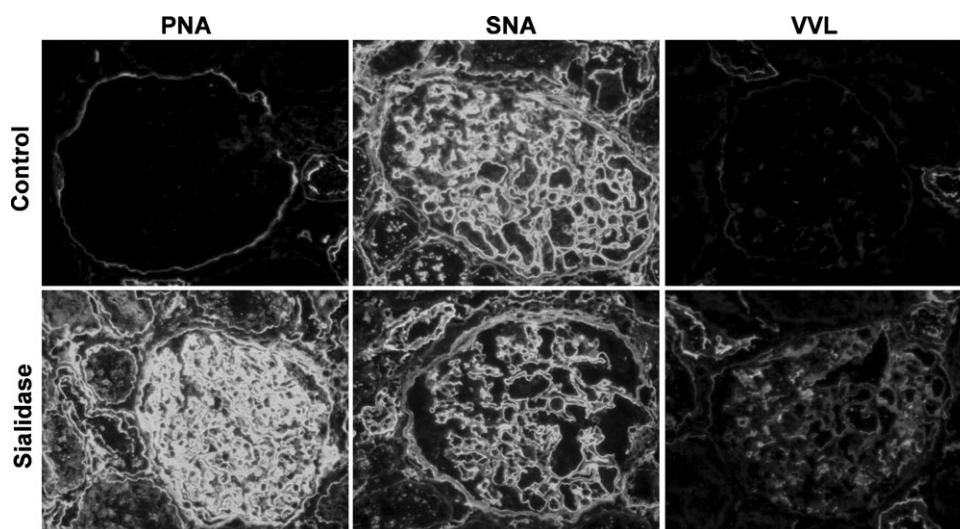
Fig. 4. Effect of *in situ* bacterial sialidase on lectin staining. Normal human kidney sections were treated with bacterial sialidase and probed with PNA and VVL (binding terminal Gal and/or GalNAc) and sambucus nigra agglutinin (SNA, specific for terminal sialic acid). After removal of terminal sialic acid, the staining of SNA is only slightly decreased, whereas the desialization is clearly apparent in the accessibility of terminal Gal and GalNAc residues by binding of PNA and VVL. The expected increased expression of PNA and VVL was not found in membranous nephropathy, which is most likely due to further deglycosylation by other enzymes or reactive oxygen species. See Table 3 for the average scores and statistics.

sections with bacterial sialidase, this was indeed the case since enhanced staining was found with PNA and VVL (Figure 4). Unexpectedly, the increased sialidase expression in MG was not accompanied with a significant increased binding of the lectins PNA and VVL. The absence of an increased glomerular staining of PNA and VVL in face of the increased sialidase expression in patients with MG may be due to a different substrate specificity of mammalian sialidase compared to bacterial sialidase, by masking of the galactosyl/*N*-acetylgalacatosamine residues by other carbohydrates, or most likely by further deglycosylation by other enzymes or reactive oxygen species.

Recently, it was clearly shown that NEU1 can be also expressed at the cell surface, where it facilitates elastic fibre assembly in the extracellular matrix [40]. Importantly, by using a newly developed LC-tandem mass spectrometry method, we could demonstrate an increased urinary sialic acid concentration in two patients with MG, but not in patients with FSGS, MCN and healthy volunteers. Presumably, the increased urinary concentration of sialic acid in these MG patients is the result of the increased sialidase activity. We postulate that neuraminidase 1 in podocytes can be expressed at the cell surface and is upregulated in MG. Sialidase then catalyses the desialization of dystroglycan, podocalyxin and other negatively charged sialoproteins resulting in podocyte foot process effacement.

It is very interesting that we did not find a decreased or altered staining for two different glyco-epitopes on α-DG, which are likely to carry the negative charge of α-DG. The expression of α-DG in the various forms of primary glomerular disease was unchanged. This finding is different from a previous report in which the expression of α-DG was found to be decreased in MCN in contrast to FSGS [21]. It was even proposed as a tool to distinguish these entities. We cannot explain these contrasting results. We used the same probes as Regele and colleagues. It must be noted that our patient group with MCN was older (mean age

60.4 versus 33.6 years). Furthermore, we used immunofluorescence rather than immunohistochemistry on paraffin sections, to avoid the procedures needed to retrieve hidden epitopes (dewaxing, rehydration, microwave exposure) and because of the higher sensitivity. In addition, at the mRNA level, the glomerular expression of α-DG was also not decreased in MCN nor in FSGS when compared with a NHK [41]. In conclusion, we did not find changes in expression of the α-DG complex in primary glomerular diseases. Therefore, the α-DG complex does not seem part of the molecular assembly responsible for the charge-dependent permeability. We were surprised by this finding, because previously we found evidence that the glycosylated part of DG was susceptible to deglycosylation by reactive oxygen species and showed a diminished expression in the adriamycin nephropathy. Recently, a podocyte-specific DG knock-out mouse was generated. In these mice, there was no proteinuria. Although preliminary, these observations do not support a primary role for DG in glomerular permselectivity [42].

We conclude that the expression of endogenous sialidase is increased in MG, which may lead to a loss of negative charge on the podocyte and this may reveal a new mechanism of podocyte foot process effacement. The expression of α- and β-dystroglycan is not altered in nephrotic and nephritic glomerular diseases and cannot be used as a tool to distinguish MCN and FSGS.

References

1. Feehally J, Floege J, Johnson RJ. *Comprehensive Clinical Nephrology*. 3rd edn. Philadelphia: Mosby Elsevier, 2007
2. Van Den Born J, Van Den Heuvel LP, Bakker MA *et al*. Distribution of GBM heparan sulfate proteoglycan core protein and side chains in human glomerular diseases. *Kidney Int* 1993; 43: 454–463
3. Haraldsson B, Nystrom J, Deen WM. Properties of the glomerular barrier and mechanisms of proteinuria. *Physiol Rev* 2008; 88: 451–487
4. Raats CJ, Van Den Born J, Berden JH. Glomerular heparan sulfate alterations: mechanisms and relevance for proteinuria. *Kidney Int* 2000; 57: 385–400
5. Kanwar YS, Farquhar MG. Detachment of endothelium and epithelium from the glomerular basement membrane produced by kidney perfusion with neuraminidase. *Lab Invest* 1980; 42: 375–384
6. Gelberg H, Healy L, Whiteley H *et al*. In vivo enzymatic removal of alpha 2→6-linked sialic acid from the glomerular filtration barrier results in podocyte charge alteration and glomerular injury. *Lab Invest* 1996; 74: 907–920
7. Miner JH. Glomerular filtration: the charge debate charges ahead. *Kidney Int* 2008; 74: 259–261
8. Morita H, Yoshimura A, Kimata K. The role of heparan sulfate in the glomerular basement membrane. *Kidney Int* 2008; 73: 247–248
9. Harvey SJ, Jarad G, Cunningham J *et al*. Disruption of glomerular basement membrane charge through podocyte-specific mutation of agrin does not alter glomerular permselectivity. *Am J Pathol* 2007; 171: 139–152
10. Chen S, Wassenhove-McCarthy DJ, Yamaguchi Y *et al*. Loss of heparan sulfate glycosaminoglycan assembly in podocytes does not lead to proteinuria. *Kidney Int* 2008; 74: 289–299
11. Van Den Hoven MJ, Wijnhoven TJ, Li JP *et al*. Reduction of anionic sites in the glomerular basement membrane by heparanase does not lead to proteinuria. *Kidney Int* 2008; 73: 278–287
12. Singh A, Satchell SC, Neal CR *et al*. Glomerular endothelial glycocalyx constitutes a barrier to protein permeability. *J Am Soc Nephrol* 2007; 18: 2885–2893
13. Vogtlander NPJ, Dijkman H, Bakker MAH *et al*. Localization of alpha-dystroglycan on the podocyte: from top to toe. *J Histochem Cytochem* 2005; 53: 1345–1353
14. Ibraghimov-Beskrovnaya O, Milatovich A, Ozcelik T *et al*. Human dystroglycan: skeletal muscle cDNA, genomic structure, origin of tissue specific isoforms and chromosomal localization. *Hum Mol Genet* 1993; 2: 1651–1657
15. Ibraghimov-Beskrovnaya O, Ervasti JM, Leveille CJ *et al*. Primary structure of dystrophin-associated glycoproteins linking dystrophin to the extracellular matrix. *Nature* 1992; 355: 696–702
16. Raats CJ, Van Den Born J, Bakker MA *et al*. Expression of agrin, dystroglycan, and utrophin in normal renal tissue and in experimental glomerulopathies. *Am J Pathol* 2000; 156: 1749–1765
17. Vogtlander NPJ, Tamboer WPM, Bakker MAH *et al*. Reactive oxygen species deglycosilate glomerular alpha-dystroglycan. *Kidney Int* 2006; 69: 1526–1534
18. Vogtlander NPJ, Visch HJ, Bakker MA *et al*. Ligation of alpha-dystroglycan on podocytes induces intracellular signaling. A new mechanism for podocyte effacement? *PLoS ONE* 2009; 4: e5979
19. Orlando RA, Takeda T, Zak B *et al*. The glomerular epithelial cell anti-adhesin podocalyxin associates with the actin cytoskeleton through interactions with ezrin. *J Am Soc Nephrol* 2001; 12: 1589–1598
20. Lai KW, Wei CL, Tan LK *et al*. Overexpression of interleukin-13 induces minimal-change-like nephropathy in rats. *J Am Soc Nephrol* 2007; 18: 1476–1485
21. Regele HM, Fillipovic E, Langer B *et al*. Glomerular expression of dystroglycans is reduced in minimal change nephrosis but not in focal segmental glomerulosclerosis. *J Am Soc Nephrol* 2000; 11: 403–412
22. Luimula P, Sandstrom N, Novikov D *et al*. Podocyte-associated molecules in puromycin aminonucleoside nephrosis of the rat. *Lab Invest* 2002; 82: 713–718
23. Andrews PM. Glomerular epithelial alterations resulting from sialic acid surface coat removal. *Kidney Int* 1979; 15: 376–385
24. Galeano B, Klootwijk R, Manoli I *et al*. Mutation in the key enzyme of sialic acid biosynthesis causes severe glomerular proteinuria and is rescued by *N*-acetylmannosamine. *J Clin Invest* 2007; 117: 1585–1594
25. Quaggin SE. Sizing up sialic acid in glomerular disease. *J Clin Invest* 2007; 117: 1480–1483
26. Skutelsky E, Hartzan S, Socher R *et al*. Modifications in glomerular polyanion distribution in adriamycin nephrosis. *J Am Soc Nephrol* 1995; 5: 1799–1805
27. Cohen-Forterre L, Mozere G, Andre J *et al*. Studies on kidney sialidase in normal and diabetic rats. *Biochim Biophys Acta* 1984; 801: 138–145
28. Cardenas A, Schadeck C, Bernard A *et al*. Depletion of sialic acid without changes in sialidase activity in glomeruli of uninephrectomized diabetic rats. *Biochem Med Metab Biol* 1991; 46: 416–421
29. Baricos WH, Cortez-Schwartz S, Shah SV. Renal neuraminidase. Characterization in normal rat kidney and measurement in experimentally induced nephrotic syndrome. *Biochem J* 1986; 239: 705–710
30. Deegens JK, Assmann KJ, Steenbergen EJ *et al*. Idiopathic focal segmental glomerulosclerosis: a favourable prognosis in untreated patients? *Neth J Med* 2005; 63: 393–398
31. Branten AJ, du Buf-Vereijken PW, Klasen IS *et al*. Urinary excretion of beta2-microglobulin and IgG predict prognosis in idiopathic membranous nephropathy: a validation study. *J Am Soc Nephrol* 2005; 16: 169–174
32. Raats CJ, Bakker MA, Hoch W *et al*. Differential expression of agrin in renal basement membranes as revealed by domain-specific antibodies. *J Biol Chem* 1998; 273: 17832–17838
33. Grootscholten C, Bajema IM, Florquin S *et al*. Interobserver agreement of scoring of histopathological characteristics and classification of lupus nephritis. *Nephrol Dial Transplant* 2008; 23: 223–230
34. Deegens JK, Steenbergen EJ, Wetzels JF. Review on diagnosis and treatment of focal segmental glomerulosclerosis. *Neth J Med* 2008; 66: 3–12
35. Holthofer H, Virtanen I. Glycosylation of developing human glomeruli: lectin binding sites during cell induction and maturation. *J Histochem Cytochem* 1987; 35: 33–37
36. Faraggiana T, Malchiodi F, Prado A *et al*. Lectin-peroxidase conjugate reactivity in normal human kidney. *J Histochem Cytochem* 1982; 30: 451–458
37. Rogerieux F, Belaise M, Terzidis-Trabelsi H *et al*. Determination of the sialic acid linkage specificity of sialidases using lectins in a solid phase assay. *Anal Biochem* 1993; 211: 200–204
38. Cohen-Forterre L, Mozere G, Andre J *et al*. Cleavage of oligosaccharides by rat kidney sialidase. Influence of substrate structure. *FEBS Lett* 1984; 173: 191–195
39. Vinogradova MV, Michaud L, Mezentsev AV *et al*. Molecular mechanism of lysosomal sialidase deficiency in galactosialidosis involves its rapid degradation. *Biochem J* 1998; 330(Pt 2): 641–650
40. Hinek A, Pshezhetsky AV, von Itzstein M *et al*. Lysosomal sialidase (neuraminidase-1) is targeted to the cell surface in a multiprotein complex that facilitates elastic fiber assembly. *J Biol Chem* 2006; 281: 3698–3710
41. Schmid H, Henger A, Cohen CD *et al*. Gene expression profiles of podocyte-associated molecules as diagnostic markers in acquired proteinuric diseases. *J Am Soc Nephrol* 2003; 14: 2958–2966
42. Harvey SJ, Miner JH. Revisiting the glomerular charge barrier in the molecular era. *Curr Opin Nephrol Hypertens* 2008; 17: 393–398