Expression of sialidase and dystroglycan in human glomerular diseases

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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 described in minimal change nephropathy (MCN), but not in focal segmental glomerulosclerosis (FSGS). This suggests 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 investigated by immunofluorescence in kidney biopsies of patients with MCN (n = 5), FSGS (n = 15), proliferative lupus nephritis (LN, n = 9), membranous glomerulopathy (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 sialidase was found in MG, accompanied with an increased urinary sialic acid concentration in two MG patients. However, 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 mechanism 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 fenestrated endothelial cells, the glomerular basement membrane (GBM) and the visceral epithelial cells or podocytes. It allows size- and charge-dependent filtration, which is disturbed in proteinuric diseases. Minimal change nephropathy (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 membranous glomerulopathy (MG). The kidney is also often involved in systemic diseases. The best-known example is systemic lupus nephritis (LN), which has a variable histology, which may include a membranous pattern of injury and/or mesangial, mesangiocapillary or extracapillary proliferation. In these glomerular diseases, the negative charge is diminished in the glomerular capillary filter, both including 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 permeability 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 possibilities have been proposed: the endothelial glycoalyx [12] and the podocyte including the slit diaphragm [3]. Since anionic charge is decreased in many glomerular diseases, we have focused 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
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 laminitin, 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 over-expressing 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 nephritis 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 glyocalyx on glomerular endothelial cells was also assigned an important role in the charge selective permeability of the glomerular filtration slit, as in vivo removal of negatively charged sialic acids results in increased permeability to albumin [12].

The crucial function of sialoglycans 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-α-N-acetylgalactosamine-2-epimerase/α-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, were used 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

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**Table 1. Antibodies, lectins and conjugates**

<table>
<thead>
<tr>
<th>Primary antibody or lectin</th>
<th>Epitope</th>
<th>Dilution primary antibody</th>
<th>Conjugates</th>
<th>Dilution secondary antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM72&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Core protein agrin (central)</td>
<td>1:400</td>
<td>Goat anti-mouse IgG1 Alexa fluor 568&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>MI 90&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Core protein agrin (N-terminal)</td>
<td>1:400</td>
<td>Goat anti-Syrian hamster IgG CY3&lt;sup&gt;c&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>IIH6&lt;sup&gt;d&lt;/sup&gt;</td>
<td>α-Dystroglycan carbohydrates</td>
<td>1:20</td>
<td>Goat anti-mouse IgG Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>VIA4.1&lt;sup&gt;d&lt;/sup&gt;</td>
<td>α-Dystroglycan carbohydrates</td>
<td>1:5</td>
<td>Goat anti-mouse IgG Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>NCL-BDG&lt;sup&gt;e&lt;/sup&gt;</td>
<td>β-Dystroglycan</td>
<td>1:10</td>
<td>Goat anti-mouse IgG Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>ShmAβDG 549–572&lt;sup&gt;e&lt;/sup&gt;</td>
<td>α-Dystroglycan core protein aa 549–572</td>
<td>1:25</td>
<td>Goat anti-sheep IgG Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:1000</td>
</tr>
<tr>
<td>Sialidase&lt;sup&gt;f&lt;/sup&gt;</td>
<td>Neu1&lt;sup&gt;f&lt;/sup&gt;</td>
<td>1:200</td>
<td>Goat anti-rabbit Alexa fluor 568&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td><em>Vicia villosa</em> lectin&lt;sup&gt;i&lt;/sup&gt; (VVL)</td>
<td>Beta-linked or Ser/Thr-linked term GalNAc</td>
<td>1:1000</td>
<td>Streptavidin Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td>Peanut agglutinin&lt;sup&gt;j&lt;/sup&gt; (PNA)</td>
<td>Galβ1–3GalNAc</td>
<td>1:1000</td>
<td>Streptavidin Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
<tr>
<td><em>Sambucus nigra</em> agglutinin (SNA)</td>
<td>Sialic acid with pre-terminal α-2,6 galactose</td>
<td>1:1000</td>
<td>Streptavidin Alexa fluor 488&lt;sup&gt;b&lt;/sup&gt;</td>
<td>1:200</td>
</tr>
</tbody>
</table>

<sup>a</sup>Own laboratory [32].
<sup>b</sup>Molecular Probes, Leiden, the Netherlands.
<sup>c</sup>Jackson, Immunoresearch Laboratories, Inc., Westgrove, Pennsylvania.
<sup>d</sup>Kevin Campbell.
<sup>e</sup>Monosan (Novocastra Laboratories Ltd, Newcastle upon Tyne, UK).
<sup>f</sup>Alexey Pshezhetsky, Université de Montreal, Service de Génétique Médicale, Département de Pédiatrie, Hôpital Sainte-Justine, Montréal, Québec, Canada [39].
<sup>i</sup>Vector Laboratories Inc., Burlington, Canada.
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-dystroglycan. This antibody barely reacts with NHK [17], but epitopes could be revealed in glomerular diseases as it is directed at the binding site of α-dystroglycan. NCLβDG 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; na, not available.

Table 3. Scores of markers in glomerular disease

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

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. NCLβDG 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.

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 α-dystroglycan.
Glomerular sialidase and dystroglycan

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 increased (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 unmask 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).

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 subtypes of mammalian sialidase, originally characterized by their localization: lysosomal neuraminidase 1 (NEU1); cytosolic neuraminidase 2; plasma membrane-associated neuraminidase 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 unmasksthe pre-terminal galactose-N-acetylgalactosamine after the removal of the terminal sialic acid [35,36]. If we treated NHK
or altered staining for two different glyco-epitopes on \( \alpha \)-dystroglycan resulting in podocyte foot process effacement. The expression of \( \alpha \)-DG is not altered in nephrotic and nephritic glomerular diseases and cannot be used as a tool to distinguish MCN and FSGS.

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**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.
Department of Nephrology of the Radboud University Nijmegen Medical Centre for her help in the 2-h urine collection of the patients. We would also like to thank Dr Dirk Lefeber of the Laboratory of Pediatrics and Neurology, Department of Laboratory Medicine, for his excellent support in the sialic acid analysis. Furthermore, we thank A van Rooij for developing the assay for urinary-free neuraminic acid. The Dutch Kidney Foundation is gratefully acknowledged for their financial support, grants C.99.1832 and C.03.2067.

Conflicts of interest statement. None declared.

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