Dystroglycan in the Diagnosis of FSGS

Giovanna Giannico,† Haichun Yang,* Eric G. Neilson,‡ and Agnes B. Fogo*†
*Department of Pathology, Vanderbilt University Medical Center, Nashville, Tennessee; †Department of Medicine, Vanderbilt University School of Medicine, Nashville, Tennessee

Background and objectives: α- and β-dystroglycan (DG), which link the actin cytoskeleton of the podocyte to the glomerular basement membrane, are maintained in FSGS but decreased in minimal change disease (MCD). Fibrosis has been linked to increased fibroblast-specific protein-1 (FSP1) and epithelial–mesenchymal transition. We studied DG, FSP1, and podocyte differentiation in FSGS variants and cases of suspected FSGS.

Design, setting, participants, & measurements: We studied renal biopsies with FSGS, not otherwise specified (NOS), tip lesion, or collapsing variants (COLL), versus secondary FSGS or cases without segmental sclerotic lesions where a diagnosis of MCD versus FSGS could not be established (undefined [UNDEF]) and compared the expression of DG, FSP1, and podocyte Wilms’ tumor antigen (WT1).

Results: WT1 is markedly decreased in NOS versus normal and correlates with the extent of sclerosis. α- and β-DG are maintained in most primary and secondary FSGS cases. In contrast, α-DG is significantly decreased in UNDEF, supporting a diagnosis of MCD. Furthermore, follow-up shows remission or decreased proteinuria in four of six of these UNDEF cases in response to therapy. Interstitial FSP1 is numerically highest in COLL but is only rarely found in tubules or podocytes in any other forms of FSGS.

Conclusions: We conclude that increased FSP1 may be a marker of the aggressive course of collapsing FSGS. Furthermore, DG staining is a useful adjunct to assist in distinction of FSGS versus MCD in biopsies without defining lesions.


I diopathic FSGS comprises clinically and pathologically heterogeneous entities with variable renal outcomes. We recently proposed a morphologic classification of FSGS with possible prognostic significance (1). Underlying podocyte injury is common to all types of glomerular diseases with massive proteinuria. In some conditions, such as minimal change disease, proteinuria and podocyte cellular alterations are steroid responsive and reversible, whereas in other conditions, such as FSGS, they are more resistant, and chronic kidney disease often ensues. Podocyte injury is postulated as a central mechanism in the development of glomerulosclerosis. Primary podocyte injury is also postulated to play a central role in the idiopathic FSGS. Although the nature of the injurious factors remains to be discovered, recent studies point to polymorphism of myosin heavy chain (MYH9) as a potential contributor to disease in some populations, i.e., African Americans (2).

Podocytes are highly specialized cells whose functions include support of the glomerular capillaries, synthesis of glomerular basement membrane (GBM), and regulation of permselectivity. These complex functions depend on a highly differentiated and unique cytoarchitecture. Podocyte injury may occur as a consequence of genetic mutation, immunological injury, viral infection, drugs (such as heroin, lithium, pamidronate, and IFN), or abnormal hemodynamic forces. Our knowledge of the podocyte has greatly increased over the past decade. α- and β-dystroglycan (DG) are matrix receptors, serving as anchoring mechanisms to the GBM (3). The DG α-chain contains a polyanionic-binding site for the cationic laminin globular-binding domain common to several matrix proteins, such as laminin, agrin, perlecan, and proteoglycans (4–9). The noncovalently attached β-chain links the DG complex to the actin cytoskeleton (10,11). Regele et al. (12) showed in a small group of patients that α- and β-DG are maintained in FSGS but decreased in minimal change disease (MCD). Further injury to podocytes with reactive oxygen species (ROS) or protamine sulfate disrupted DG attachment, leading to podocyte effacement (13). Whether DG expression aids in differentiating types of FSGS or distinguishing MCD from likely unsampled FSGS in small biopsies without diagnostic sclerosis has not been determined. The mature podocyte also shows expression of several key factors that maintain a mature phenotype. Among these, Wilms tumor antigen (WT1) is a zinc-finger transcription factor that regulates proliferation. Failure of metanephric development has been reported in WT1-deficient mice (14). WT1 is widely expressed in developing glomerular progenitor cells but becomes restricted to podocytes as the glomerulus matures. Mutations of the WT1 gene are implicated in special forms of nephrotic syndrome, such as Denys-Drash syndrome and Frasier syndrome (15). Loss of podocyte expression of WT1 in primary FSGS has been observed (16).

The diagnostic and pathogenic differentiation of MCD from FSGS is also theoretically illuminated by expression of key...
profibrotic factors. Among these are fibroblast-specific protein 1 (FSP1), a cytoskeleton-associated, calcium-binding protein that is normally expressed in fibroblasts, that may be a key marker in the process of epithelial–mesenchymal transition, a process that may contribute to fibrosis (17).

In this study, we test the hypotheses that different histologic patterns of injury in FSGS correlate with changes in podocyte differentiation, as expressed by WT1 and podocyte-matrix anchoring, as expressed by α- and β-DG, and whether this expression aids in diagnosis. Furthermore, we assess a possible relationship between changes observed in such marker expression and renal outcome in patients with different forms of FSGS. Last, we examine whether FSP1 expression is informative in predicting fibrosis and cause of chronic kidney disease.

Materials and Methods

Case Selection

Renal biopsies diagnosed at Vanderbilt University Medical Center from 2000 to 2004 were reviewed, and cases with sufficient material (at least five glomeruli) for further study were selected and compared with normal control kidneys (NL; n = 4). Normal control kidneys showed no evidence of abnormalities by light, immunofluorescence, and electron microscopy. Cases included primary FSGS, NOS variant (NOS; n = 11), tip lesion variant (TIP; n = 8), idiopathic collapsing variant (COLL; n = 5), secondary FSGS (SEC; n = 8), and cases with foot process effacement (FPE) without segmental lesions where the differential diagnosis was MCD versus unsampled FSGS (UNDEF; n = 10). Cases with hilar or cellular FSGS were not included. Cases without segmental sclerosis but with significant glomerular hypertrophy, a feature we have previously linked to evolution to overt FSGS, were not included (18). Primary FSGS was defined by exclusion of other primary glomerular diseases and absence of other morphologic signs of a secondary cause of segmental sclerosis, such as disproportional severe vascular sclerosis, periglomerular fibrosis, surrounding nonsclerotic glomeruli, lamina rara interna expansion (a sign of hypertension-associated and/or chronic endothelial injury) by electron microscopy, limited FPE; lesions indicative of arteriomegathro sclerosis; or geographic pattern scarring with periglomerular fibrosis, lesions indicative of chronic pyelonephritis/relux nephropathy). FSGS cases showed the presence of at least one glomerulus with a defining lesion as defined in the Columbia FSGS classification (1). Briefly, FSGS NOS was defined by the presence of a segmental lesion with increased matrix and obliteration of capillary lumina. Tip lesion variant of FSGS was defined by at least one segmental lesion involving the tip domain of the glomerular tuft at the tubular lumen and the absence of collapsing or hilar lesions. Collapsing variant of FSGS was defined by at least one glomerulus with glomerular tuft collapse and overlying podocyte hypertrophy and hyperplasia. Cases with nephrotic or subnephrotic proteinuria with no segmental sclerosing lesions and extensive FPE and absence of other defining lesions on renal biopsy were classified as undefined, with a renal biopsy differential of MCD versus unsampled FSGS. Secondary forms of FSGS included cases with glomerular scarring secondary to hypertension (n = 7) and chronic reflux/pyelonephritis (n = 1). Other causes of secondary FSGS, such as obesity, diabetes, reduced renal mass, or viral-induced podocyte injury, namely HIV-associated nephropathy, were not included in this study.

Clinical Parameters

Clinical markers and outcomes were compared, including age, gender, race, systolic and diastolic BP, serum creatinine, proteinuria, therapy, and follow-up. In adults (>16 yr of age), complete remission was defined as reduction of proteinuria to ≤0.20 g/d and serum albumin >3.5 mg/dl. Partial remission was defined as reduction of proteinuria to between 0.21 and 3.4 g/d and/or decrease in proteinuria of ≥50% from baseline (19). Renal insufficiency was defined as serum creatinine ≥1.2 mg/dl. In the pediatric population (≤16 yr of age), lack of remission was defined as proteinuria >40 mg/m²/h (19); renal insufficiency was defined according to the Schwartz equation (20,21).

Renal Biopsy Study

Renal biopsies were processed by standard techniques for light (LM), immunofluorescent (IF, stained for IgG, IgM, IgA, C3, C1q, kappa and lambda light chain), and electron microscopy. For LM evaluation, serial sections were stained with hematoxylin and eosin, periodic acid-Schiff reagent, and methenamine silver stains. Number of glomeruli for LM analysis was on average 17 for NOS (range, 7 to 47), 20 for tip (range, 4 to 35), 13 for UNDEF (range, 4 to 23), 20 for COLL (range, 13 to 33) and 14 for SEC (range, 7 to 34).

Two-micrometer renal biopsy sections were stained for α-DG, β-DG, and FSP1. Antigen retrieval was performed by microwaving the sections for 30 min in 10 mM citrate buffer, pH 6.0, for α- and β-DG and for 2 min for FSP1. The tissue was washed, and after quenching of endogenous peroxidase activity with 3% (vol/vol) H2O2 in methanol for 10 min, blocked for 30 min in PBS 0.4% Triton-Parablock and incubated with polyclonal antibodies directed against β-DG (1:100; Novocastra, Newcastle, UK) or α-DG (1:100; Upstate, Lake Placid, NY) overnight at 4°C or against FSP1 (polyclonal rabbit anti-FSP1, 1:200) at 37°C for 1 h in a humidified chamber. Secondary antibody IgG antismouse (1:200; Vectastain, Burlingame, CA) for α-DG and goat anti-rabbit (1:50; Southern Biotechnology Associates, Birmingham, AL) for FSP1 were added, incubated for 30 min followed by anti-mouse avidin-biotinylated complex (1:10; Vectastain) for α- and β-DG and 3,3’-diaminobenzidine for FSP1, and subsequently counterstained with hematoxylin (Sigma-Aldrich, St. Louis, MO).

For WT1, antigen retrieval was performed by steaming the sections for 35 min in 10 mM citrate buffer, pH 6.0, quenched with 3% (vol/vol) H2O2 for 5 min, incubated with a monoclonal antibody directed against WT1 (1:100; Dako, Carpinteria, CA) for 30 min, followed by sequential 10-mins incubation with a biotinylated link antibody containing anti-rabbit and anti-mouse immunoglobulins and peroxidase-labeled streptavidin, according to the LSAB2 System (Dako). Peroxidase-stained sections were incubated for 5 min with 3,3’-diaminobenzidine and subsequently counterstained with Gill 2 hematoxylin (Thermo Fisher Scientific, Waltham, MA).

Positive controls included normal kidney for α- and β-DG, fibrotic kidneys for FSP1, and Wilms’ tumor for WT1. Negative controls with incubation of sections with preimmune serum and omission of the primary antibody showed no staining.

Immunohistochemistry Scoring

Immunostaining was evaluated by LM in a blinded fashion. Resident glomerular cells were identified by morphology and anatomical location. For α- and β-DG, GBM staining for each glomerulus was scored on a 0 to 3+ scale: 0, <5% staining; 1+, 5 to 10% staining; 2+, 10% to 25% staining; and 3+, >25% staining of GBM. WT1 staining was scored for each glomerulus on a scale from 0 to 3+: 0, no staining; 1+, ≤50% of podocyte staining; 2+, >50% staining; 3+, staining of all podocytes. Tubular, interstitial, and vascular FSP1 staining was scored as follows: 0, absent staining; 1+, moderately strong staining in <25% of cells; 2+, strong staining in 25 to 50% of the cells; and 3+, very strong staining in >50% of cells.
Statistical Analyses
Results are expressed as mean ± SE. Continuous variables were assessed by ANOVA followed by Tukey test. Nonparametric data were assessed by Kruskal-Wallis test followed by Dunn’s test. The Spearman correlation test for nonparametric data was used to evaluate the strength of association between FSP1 expression and degree of interstitial fibrosis in cases of COLL FSGS. P < 0.05 was considered significant.

Results
Clinical Characteristics at Biopsy
Forty-two patients were studied, including 11 NOS, 8 TIP, 5 idiopathic COLL, 10 UNDEF, and 8 SEC (Table 1). TIP FSGS patients presented with more severe nephrotic syndrome versus FSGS NOS, as shown in previous studies (22,23), and were mostly Caucasian, contrasting FSGS NOS. Collapsing FSGS patients also had more severe nephrotic syndrome and renal insufficiency than other groups. As expected, patients with SEC FSGS had the highest average age and the lowest level of urinary protein excretion.

Table 1. Clinical findings at time of renal biopsy

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>n</th>
<th>Age (yr)</th>
<th>Sex (M/F)</th>
<th>Race (C/AA)</th>
<th>SBP (mmHg)</th>
<th>DBP (mmHg)</th>
<th>Serum creatinine (mg/dl)</th>
<th>Proteinuria (g/24 h)</th>
</tr>
</thead>
<tbody>
<tr>
<td>NOS</td>
<td>11</td>
<td>23 ± 5 (3-55)</td>
<td>5/6</td>
<td>7/4</td>
<td>131.7 ± 5.8</td>
<td>79.4 ± 4.1</td>
<td>0.9 ± 0.2</td>
<td>5.8 ± 1.2</td>
</tr>
<tr>
<td>TIP</td>
<td>8</td>
<td>42 ± 8 (12-73)</td>
<td>4/4</td>
<td>6/1</td>
<td>133.6 ± 7.0</td>
<td>83.0 ± 6.2</td>
<td>1.4 ± 0.3</td>
<td>9.6 ± 2.9</td>
</tr>
<tr>
<td>COLL</td>
<td>5</td>
<td>38 ± 11 (17-27)</td>
<td>2/3</td>
<td>3/2</td>
<td>141.6 ± 12.5</td>
<td>87.6 ± 5.0</td>
<td>2.5 ± 0.7</td>
<td>8.4 ± 2.0</td>
</tr>
<tr>
<td>UNDEF</td>
<td>10</td>
<td>45 ± 9 (5-74)</td>
<td>9/1</td>
<td>9/1</td>
<td>142.0 ± 7.0</td>
<td>81.0 ± 3.7</td>
<td>1.0 ± 0.6</td>
<td>7.6 ± 2.4</td>
</tr>
<tr>
<td>SEC</td>
<td>8</td>
<td>52 ± 5 (35-75)</td>
<td>6/2</td>
<td>3/4</td>
<td>130.9 ± 3.8</td>
<td>77.0 ± 4.1</td>
<td>1.9 ± 0.2</td>
<td>3.5 ± 0.9</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM (range).

α- and β-DG Expression
Normal control kidneys showed α- and β-DG expression along the GBM, tubular basement membranes of proximal tubules, and vascular smooth cells, as previously reported (1.00 ± 0.00) (12). β-DG was expressed along the GBM in a similar fashion in all of the FSGS subgroups studied (NOS, 0.98 ± 0.15; TIP, 0.95 ± 0.04; COLL, 1.00 ± 0.00; SEC, 0.92 ± 0.08; UNDEF, 1.00 ± 0.00), with numerically decreased expression in areas of segmental sclerosis and within the tip lesion (Figure 1). In contrast, α-DG was significantly decreased in UNDEF (0.33 ± 0.09; P < 0.05) and not significantly changed in the remaining subgroups (NOS, 0.52 ± 0.13; TIP, 0.81 ± 0.08; COLL, 0.64 ± 0.16; SEC, 0.76 ± 0.09), similarly to previously reported data (Figure 2) (12).

Importantly, these findings correlated with the clinical follow-up data (see above and Table 2), because complete remission was observed in four of six cases with follow-up of our cases without defining lesions (UNDEF) that also had decreased α-DG. Of note, complete remission was observed in most TIP lesion cases, although there was no decrease in α-DG expression. These results infer that α-DG expression could represent a marker of steroid responsiveness rather than a reliable predictor of ultimate outcome (Figure 3). These results also suggest that UNDEF cases with good outcome and low α-DG, thus presumed to be MCD, are indeed different from tip lesion cases that maintain α-DG staining.

WTI
WTI was strongly expressed in podocytes of normal kidneys and similarly expressed in glomeruli of TIP, UNDEF, and SEC FSGS variants (Table 3, Figure 4). In contrast, WTI expression was significantly decreased in the NOS variant of FSGS (P < 0.05), which tended to correlate with the extent of global and segmental sclerosis (Table 3). WTI was numerically decreased in COLL variant of FSGS, as previously reported (24).

FSP1
FSP1 was not expressed in normal control kidneys (0.0 ± 0.0) and was moderately expressed within the interstitium of control kidneys with fibrosis (2.50 ± 0.00). Interstitial FSP1 was numerically increased in all FSGS types, contrasting no staining in UNDEF cases (NOS, 0.34 ± 0.17; TIP, 0.50 ± 0.24; SEC, 0.43 ± 0.17; UNDEF, 0.00 ± 0.00) or normal control. Interstitial FSP1 was numerically highest in COLL (0.70 ± 0.30, P = 0.093 versus...
Table 2. Clinical data at time of follow-up

<table>
<thead>
<tr>
<th></th>
<th>NOS</th>
<th>TIP</th>
<th>COLL</th>
<th>UNDEF</th>
<th>SEC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Follow-up available (N)</td>
<td>7/11</td>
<td>6/8</td>
<td>3/5</td>
<td>6/10</td>
<td>6/8</td>
</tr>
<tr>
<td>Serum creatinine (mg/dl)</td>
<td>2.5 ± 1.6</td>
<td>1.2 ± 1</td>
<td>2.5 ± 1.6</td>
<td>1.2 ± 0.4</td>
<td>1.9 ± 0.3</td>
</tr>
<tr>
<td>Proteinuria (g/24 h)</td>
<td>2.1 ± 0.8</td>
<td>1.4 ± 1.6</td>
<td>13.0 ± 6.0</td>
<td>0.9 ± 0.2</td>
<td>2.3 ± 0.5</td>
</tr>
<tr>
<td>Complete remission</td>
<td>0</td>
<td>4</td>
<td>0</td>
<td>4</td>
<td>0</td>
</tr>
<tr>
<td>Partial remission</td>
<td>5</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>2</td>
</tr>
<tr>
<td>No remission</td>
<td>2</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td>4</td>
</tr>
<tr>
<td>Follow-up (mo)</td>
<td>17.6 ± 5.5</td>
<td>15.5 ± 2.8</td>
<td>19 ± 9.2</td>
<td>22.2 ± 4.8</td>
<td>36.3 ± 5.8</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM (range).

Discussion

The term FSGS encompasses numerous histologic variants that show different clinical behavior (22). The collapsing variant of FSGS has a clinically aggressive course, with rapid progression to renal failure. In contrast, the tip lesion behaves indolently with overall excellent prognosis.

In cases of nephrotic syndrome with extensive FPE, but with a small specimen without diagnostic segmental sclerosing lesions, one cannot exclude unsampled FSGS. Thus, the morphologic differential diagnosis of FSGS or MCD may be very difficult. In some patients, large biopsies may show apparent MCD, i.e., lack of segmental sclerosis at first biopsy. However, subsequent course and rebiopsy showed that the ultimate process indeed is FSGS in some of these patients (18). Whether this represents a sclerosing process from the onset or transition from MCD to FSGS is controversial. The recurrence of FSGS in the transplant is manifested by early complete FPE preceding by weeks overt sclerosis with poor response to treatment, suggesting that some podocyte injuries indeed are programmed for
progressive sclerosis leading to FSGS from the onset, whereas other injuries are less severe in that only proteinuria ensues and the lesion is steroid responsive, suggesting MCD (25). Furthermore, most recurrent cases showed fidelity of histologic phenotype, further supporting inherent pathogenetic mechanisms leading to specific types of FSGS (25). The presence of glomerular hypertrophy in patients with initial diagnosis of apparent MCD predicts an increased risk of subsequently developing overt FSGS (18). More recent molecular studies also support that MCD and FSGS differ from the onset (26). Thus, the definition of patterns of marker expression may aid in the distinction of MCD from FSGS.

In our study, we sought to identify the relationship of different subsets of FSGS, MCD, and secondary forms of FSGS. We found that β-DG is similarly expressed along the GBM in the different subgroups of FSGS and control. However, α-DG is significantly reduced in many cases without defining sclerotic lesions. Interestingly, this finding correlates with the clinical follow-up data (see Table 2), because complete remission was observed in four of these six cases with follow-up, suggesting the diagnosis may have been MCD. These data are in agreement with previous studies (12), supporting an association between decreased expression of DG and MCD. Our patients with tip lesion variant of FSGS showed complete or partial remission in 50% of cases, similar to previous studies (22,23,27), which showed 56 to 75.8% remission in tip lesions. A similar rate of complete remission was observed in TIP lesion and UNDEF cases, although these groups showed different α-DG expression. These data suggest that α-DG expression may represent a marker of steroid responsiveness rather than a reliable predictor of outcome (Figure 3). No significant change in glomerular α- and β-DG expression compared with normal was observed in the TIP lesion subgroup, supporting a pathogenesis different from MCD, where these markers are decreased (Figure 2).

WT1 expression is significantly reduced in the NOS variant cases of FSGS and correlates with the extent of sclerosis (Table 3). WT1 also tends to decrease in patients with collapsing variant of FSGS, as reported in previous studies (24). However, WT1 expression is lower in NOS compared with collapsing variant, even in the presence of a comparable amount of sclerosis in both diseases, perhaps related to loss of podocytes in sclerotic segments.

We also examined FSP1 expression, a marker of epithelial–mesenchymal transition, in tubules and interstitium, and any possible link with specific forms of FSGS. FSP1 in human IgA nephropathy is a marker associated with fibroblast accumulation and chronicity (28). In our study, FSP1 expression is numerically increased within tubules and interstitium in collapsing FSGS but has less intensity in other forms of FSGS, suggesting a possible involvement of this protein in the pathophysiology of the more aggressive course of collapsing FSGS. We speculate that the lack of correlation with existing interstitial fibrosis could reflect small samples or possibly FSP1 expression could precede fibrosis.

### Table 3. WT-1 distribution

<table>
<thead>
<tr>
<th>Diagnosis</th>
<th>%GS</th>
<th>%SS</th>
<th>WT-1 score</th>
</tr>
</thead>
<tbody>
<tr>
<td>NL CONT</td>
<td>0.0</td>
<td>0.0</td>
<td>3.00 ± 0.00</td>
</tr>
<tr>
<td>NOS</td>
<td>11.7</td>
<td>6.5</td>
<td>1.66 ± 0.37a</td>
</tr>
<tr>
<td>TIP</td>
<td>1.1</td>
<td>2.3</td>
<td>2.55 ± 0.32</td>
</tr>
<tr>
<td>UNDEF</td>
<td>1.9</td>
<td>0.0</td>
<td>2.52 ± 0.24</td>
</tr>
<tr>
<td>COLL</td>
<td>9.2</td>
<td>10.5</td>
<td>2.26 ± 0.34</td>
</tr>
<tr>
<td>SEC</td>
<td>9.3</td>
<td>5.6</td>
<td>2.46 ± 0.15</td>
</tr>
</tbody>
</table>

Data are shown as mean ± SEM. *P < 0.05 versus normal control.

### Figure 4. WT-1 expression in normal control (A), FSGS NOS (B), tip lesion variant (C), and undefined cases (D) (magnification, ×400, ×200, ×100, and ×200, respectively).

### Figure 5. FSP1 expression. (I) FSP1 expression in normal control (A) and collapsing variant of FSGS (B) (magnification, ×100). (II) FSP1 distribution in different FSGS subclasses.
In summary, our study showed that β-DG is maintained in most FSGS cases. α-DG is also maintained in nonsclerotic segments of FSGS NOS cases and is significantly decreased in cases without defining lesions, linked to remission in some of these cases. WTI is markedly decreased in NOS versus normal and correlates with extent of sclerosis. Interstitial FSP1 is increased only in collapsing FSGS.

Based on these findings, we postulate that α-DG staining is a useful adjunct marker to assist in distinction of likely FSGS versus MCD in small biopsies in nephrotic patients with extensive FPE without defining lesions. We speculate that FSP1 may be a marker of the aggressive course of collapsing FSGS.

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Disclosures
None.

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See related editorial, “Dystroglycan in the Molecular Diagnosis of the Podocytopathies,” on pages 1696–1698.