Characterization of glomerular diseases using proteomic analysis of laser capture microdissected glomeruli

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The application of molecular techniques to characterize clinical kidney biopsies has the potential to provide insights into glomerular diseases that cannot be revealed by traditional renal pathology. The present work is a proof-of-concept approach to test whether proteomic analysis of glomeruli isolated from clinical biopsies by laser capture microdissection can provide unique information regarding differentially expressed proteins relevant to disease pathogenesis. The proteomes of glomeruli isolated by laser capture microdissection from biopsies of normal kidneys (living-related donor kidneys) were compared with those from patients with diabetic nephropathy, lupus nephritis, and fibronectin glomerulopathy. Glomerular proteins were extracted, trypsin digested, and subjected to liquid chromatography–tandem mass spectrometry for identification and quantitation. Relative to normal glomeruli, all disease-associated glomeruli showed an increased presence of complement components, a marked decline in podocyte-associated proteins, and a decrease in proteins associated with cellular metabolism. Additionally, fibronectin glomerulopathy glomeruli differed from all the other glomeruli because of a significant accumulation of fibronectin and fibulin. This study demonstrates that our method acquires reproducible and quantitative proteomic information from laser capture microdissection isolates that can be used to characterize the molecular features of glomerular diseases.

The application of molecular techniques such as genomics, proteomics, or metabolomics to clinical kidney biopsies has the potential of providing significantly more new and unique information regarding glomerular diseases than traditional renal pathology using microscopy. New insights could be relevant to glomerular disease pathogenesis, novel therapeutic targets, or inter-patient variability. While there have

been several reports on the use of laser capture microdissection in the analysis of glomerular diseases, these investigations mainly focused on changes in gene expression in specific kidney compartments.[1–6] A small number of studies have tested the feasibility of proteomic analysis of isolated glomeruli in animal models[7,8] or human tissue.[9–11] Only one investigation showed that laser capture microdissection glomeruli from a clinical kidney biopsy specimen yielded sufficient material to create a robust proteome. However, none of the proteins was further characterized.[10]

The present work was undertaken to more clearly establish whether proteomic analysis of laser capture microdissection glomeruli from clinical kidney biopsies can be used to identify differentially expressed glomerular proteins that may inform disease

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pathogenesis. For this proof-of-concept study, we feature an examination of laser capture microdissection glomeruli from patients with diabetic nephropathy, lupus nephritis, and fibronectin glomerulopathy. Fibronectin glomerulopathy is a rare disease with large, proteinaceous glomerular deposits, which to date have only been assessed by traditional immunohistochemistry. Thus, it is an ideal clinical model to evaluate the extent to which analysis by proteomics can exceed that of immunohistologic methods.

Fibronectin glomerulopathy is a familial glomerulopathy characterized clinically by proteinuria, microscopic hematuria, hypertension, and progressive renal dysfunction, which can lead to end-stage renal disease.[12] An autosomal dominant inheritance pattern with age-related penetrance affecting both genders has been demonstrated.[13,14] Histologically, fibronectin glomerulopathy is characterized by lobular glomerular enlargement, obliteration of capillary loops, and mesangial expansion due to accumulation of acellular material. These deposits are large homogenous to finely granular electron dense masses, usually with no distinctive fibrillary substructure. By immunohistochemical staining, the glomerular material stains for fibronectin[15] and only weakly or not at all for IgG, complement products, or other matrix components like laminin and collagen type IV.[15] Using antibodies to plasma-derived and cell-derived fibronectin, it has been reported that the fibronectin in the deposits is mainly plasma derived.[15,16] Therefore, it has been postulated that fibronectin glomerulopathy is a primary mesangiopathic glomerulopathy that is caused by abnormal plasma fibronectin deposition, and subsequent disruption of the glomerular architecture and filtration barrier leading to glomerular proteinuria and loss of glomerular filtration rate.

However, fibronectin deposition is not specific for fibronectin glomerulopathy. Intra-glomerular fibronectin can be found in other kidney diseases, such as diabetic nephropathy and lupus nephritis.[17–19] These findings suggested that comparing glomerular protein, expression of fibronectin glomerulopathy to that of normal kidneys, and kidneys with diabetic nephropathy and lupus nephritis would be a good test for demonstrating the capability of using laser capture microdissection in combination with liquid chromatography–tandem mass spectrometry to characterize kidney diseases in greater detail. The approach used here revealed anticipated differences as well as unexpected similarities between disease states, and also highlighted potential pathogenic mechanisms not previously known or considered for these diseases.

Materials and methods

Kidney Tissue

Baseline allograft biopsies from living donor renal allograft recipients ($n = 3$) were used as normal controls. Glomerular disease biopsies included diabetic nephropathy ($n = 2$), lupus nephritis proliferative class IV ($n = 3$), and pure membranous class V ($n = 2$), and two patients from a family known to have fibronectin glomerulopathy. The kidney biopsy findings of this family have been previously reported.[20] One biopsy was from an affected native kidney, while the second biopsy was from a living-unrelated donor renal allograft that developed recurrent disease 3 years post-transplant. The study was approved by the Ohio State University Institutional Review Board (Protocol number 2009H0179).

Laser Capture Microdissection and Mass Spectrometry

Laser capture microdissection was performed using the laser microdissection system from PALM Technologies (Carl Zeiss MicroImaging GmbH, Munchen, Germany) containing a PALM MicroBeam and Robo-Stage for high-throughput sample collection and PALM RoboMover (PALM Robo software, Version 2.2). Typical settings used for laser cutting were UV-Energy of 52 and UV-Focus of 79–81. Laser capture microdissection was done using snap frozen tissue embedded in optimum cutting temperature medium at the time of the biopsy. The frozen tissue blocks were cut into 10 μm sections, mounted on a thermoplastic (polyethylenenapthalate) covered glass slide (Carl Zeiss MicroImaging) and kept at -80°C until use. When needed, sections were briefly thawed and immediately fixed in ice cold 70% ethanol and rinsed in deionized water for 1 min. After the water rinse, the wet sections were stained briefly (5–10 s) with hematoxylin, immediately rinsed, and then dehydrated in graded ethanol. The dehydrated slides were allowed to air dry for immediate capture on the PALM technology microdissection system.

Sections were cut and captured under a $\times 10$ ocular lens. Fifteen 10 μm sections from each clinical biopsy case were utilized, and each section contained an average of 10 glomeruli. The glomeruli were isolated by dissecting around Bowman's capsule. For each biopsy, an average of 75 glomerular cross-sections, equivalent to about 7000 cells were captured. Cut elements were catapulted into 25 μl of 0.5% Rapigest (Waters Corporation, MA, USA), resuspended in 50 mM NH_3HCO_3, collected in a 0.2-ml tube, and held at -80°C until protein retrieval.

For protein retrieval, samples that had been stored at -80°C were thawed briefly and sonicated (in the original 0.2 ml tube) on the low power setting for a total instrument time of 60 s with a 3 s on 3 s off time interval using a Diagenode Bioruptor (model UCD-200, NJ, USA). The samples were placed in a boiling water bath for 5 min and cooled to room temperature. Trypsin was added in a ratio of 1:30 trypsin:protein assuming ~ 2 μg retrieved protein/10 000 isolated cells. After overnight incubation at 37°C with gentle shaking, formic acid was added to a final concentration of 30% and the suspension was

incubated for 30 min at 37°C to precipitate the Rapigest. The extracts were dried and the protein digests were resuspended in 20 μl of a solution of 2% acetonitrile with 0.1% formic acid and sonicated for 1–2 min in a water bath sonicator at 4°C to ensure peptide solubilization. Peptide/protein concentration was obtained at 280 nm absorbance using 1 μl of sample on a Nanodrop ND-1000 spectrometer.

For mass spectrometry analysis, peptides were separated by reversed-phase HPLC (Dionex Ultimate 3000 capillary/nano HPLC system, Dionex, CA, USA) and mass analyzed with a Thermo Fisher LTQ Orbitrap XL (CA, USA), equipped with micro/ nanospray ionization sources (Michrom Bio-resources, Auburn, CA, USA). HPLC separations were carried out at a flow rate of 2 μl/min on a 0.2 mm × 150 mm C18 column (5 μm, 300 Å; Michrom Bio-resources). The mobile phases consisted of HPLC grade water and acetonitrile with formic acid added as ion-pairing reagent. A 10–32% gradient was run over 5 h followed by a 32–90% gradient run over 40 min. The heated capillary temperature and electrospray voltage were set at 175°C and 2.0 kV, respectively. Protein identifications were obtained using the MassMatrix search engine.[21–24] An average of 350 proteins were identified per biopsy, with a range of 250–450 proteins.

Immunoperoxidase Staining

Immunoperoxidase staining with a monoclonal antibody to fibulin-1 (Santa Cruz Biotechnology, CA, USA) and a polyclonal antibody to podocin (Alpha Diagnostic Intl, TX, USA) was performed (dilution 1:100 and 1:40, respectively) on formalin-fixed paraffin-embedded tissue sections of the fibronectin glomerulopathy biopsies, and additional cases of diabetic nephropathy and proliferative class IV lupus nephritis (different from the cases used for mass spectrometry). Fibronectin immunostaining was performed in the laboratory of the late Dr Andrew Herzenberg, Toronto, Canada). The antibody used was a mouse monoclonal antibody directed to the N-terminal of fibronectin. Control tissues used to validate the antibodies were normal kidney, aorta, placenta, and blood vessel walls in the gastrointestinal tract.

DNA Sequencing

Genomic DNA samples were sequenced for fibronectin gene coding region exons 19, 36, and 37, and the complete coding region, including intron-exon boundaries for the fibulin-1 gene. Genomic DNA was extracted from peripheral blood leukocytes using Gentra Puregene Blood Kits (Qiagen, CA, USA) or frozen serum using the QIAamp Investigator kit (Qiagen). DNA amplification was carried out by polymerase chain reaction (PCR). Oligonucleotide primers (Invitrogen, CA, USA) were specifically designed to flank fibronectin exons 19, 36, and 37, and fibulin-1 exons 1–20, according to the gene bank published sequences. Primer sequences are available upon request. PCR products ranged in size from 262 to 983 basepairs.

PCR products were checked on 2% agarose gel to confirm a single product for direct sequencing with BigDye Terminator Cycle Sequencing Chemistry (Applied Biosystems, CA, USA) using the same PCR primers. Sequencing was performed by 3730 DNA Analyzer by Applied Biosystems.

Data and Statistical Analysis

Protein quantitation was performed by analysis of peptide spectral counts for each protein group identified.[25–27] The MassMatrix protein identification search results were regrouped by use of an in-house developed application to create a parsimonious protein list.[28] This application regroups protein identifications based on common peptides observed in all sample data sets. Hierarchical clustering of the total peptide matches for each protein group was then performed using Cluster 3.0. The Ingenuity Pathway Analysis tool was used to examine glomerular molecular pathways based on identified proteins (Ingenuity Systems; http://www.ingenuity. com). To evaluate statistically significant difference in protein abundances, the MS/MS data for each sample classification were merged, analyzed by MassMatrix and combined into a parsimonious protein list. The resulting peptide spectral counts for each protein were then normalized by linear scaling such that the total spectral counts were equal. Sample classes were then evaluated pairwise using the G-test to determine if differences in spectral counts were significant.[29]

Results

The pedigree of the fibronectin glomerulopathy family is shown in Figure 1a, and the two family members whose biopsies were studied by laser capture microdissection/proteomics are indicated. It has previously been shown that several fibronectin glomerulopathy kindreds display an A→G single-nucleotide polymorphism in heparin-binding domain III of exon 19 of the fibronectin gene.[30] This results in a tyrosine→cysteine amino-acid change, and may provide a genetic predisposition for fibronectin glomerulopathy. Exon 19 of the fibronectin gene from our patients was sequenced and showed this same heterozygous A→G single-nucleotide polymorphism (Figure 1b). Another affected and one unaffected family member were also sequenced, and the results are shown in Figure 1a.

Renal biopsies from both fibronectin glomerulopathy patients showed prominent glomerular enlargement and diffuse acellular mesangial expansion that was periodic acid-schiff positive and
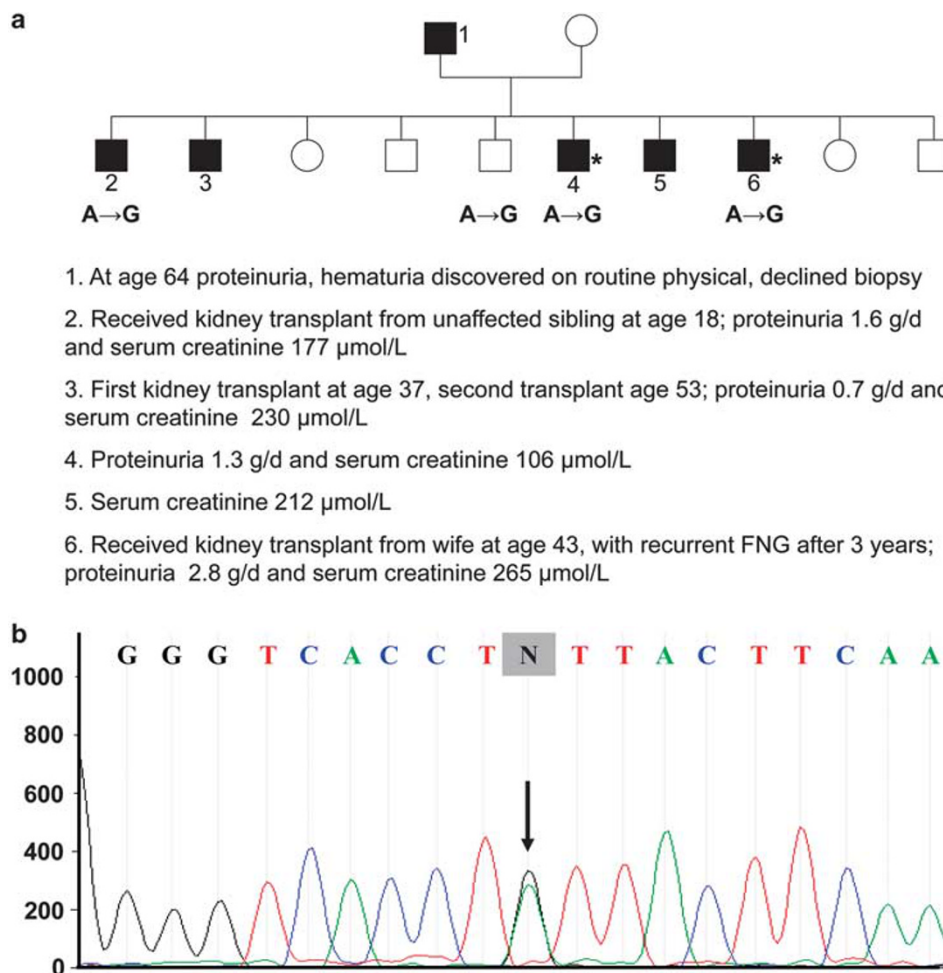
Figure 1 Pedigree and genetic analysis of a family with fibronectin glomerulopathy. (**a**) Affected members are indicated by filled symbols. Males are squares, females are circles. The patients whose biopsies were analyzed by laser capture microdissection/proteomics are indicated with an '*'. The results of genotyping the fibronectin gene *FN1* are indicated below those family members who provided DNA for analysis. (**b**) Example of DNA sequencing of one of the patients showing the common heterozygous A→G single-nucleotide polymorphism.

methenamine silver negative (Figure 2a and b). On ultrastructural examination, the glomeruli showed bulky, nondescript granular electron dense material in the mesangium and in the widened subendothelial space along glomerular capillary walls (Figure 2c). Direct immunofluorescence staining for IgG, IgM, IgA, and C3 did not reveal strong specific staining for any of these immunoreactants. The bulky glomerular deposits did, however, show staining with the antibody to fibronectin (Figure 2d).

A heat map of glomerular protein expression obtained from the liquid chromatography–tandem mass spectrometry analysis of the glomerular laser capture microdissection isolates is shown in Figure 3. The two patients with fibronectin glomerulopathy were most closely clustered, followed by the three normal controls. There was more variability among the patients with lupus nephritis and diabetic nephropathy. Compared with normal glomeruli, fibronectin glomerulopathy glomeruli displayed 133 differentially expressed proteins that achieved a predetermined level of significance (*G*-score ≥5). A subset of these differentially expressed proteins is shown in Table 1. The normalized spectral ratios show the fold-increase (or decrease) of these proteins relative to normal. Table 2 shows the raw spectral counts for each of these proteins and for each individual normal and fibronectin glomerulopathy patient. For comparison, the same proteins were also examined for differential expression relative to normal glomeruli in diabetic nephropathy and lupus nephritis (Table 1). The most striking difference between fibronectin glomerulopathy and normal glomeruli was the extensive level of fibronectin expression in the affected glomeruli. Diabetic nephropathy and lupus nephritis did not show any increase in glomerular fibronectin over control. Additionally, fibulin-1 and fibulin-5 were highly abundant in fibronectin glomerulopathy, and were not found or were minimally detected in normal glomeruli, or glomeruli
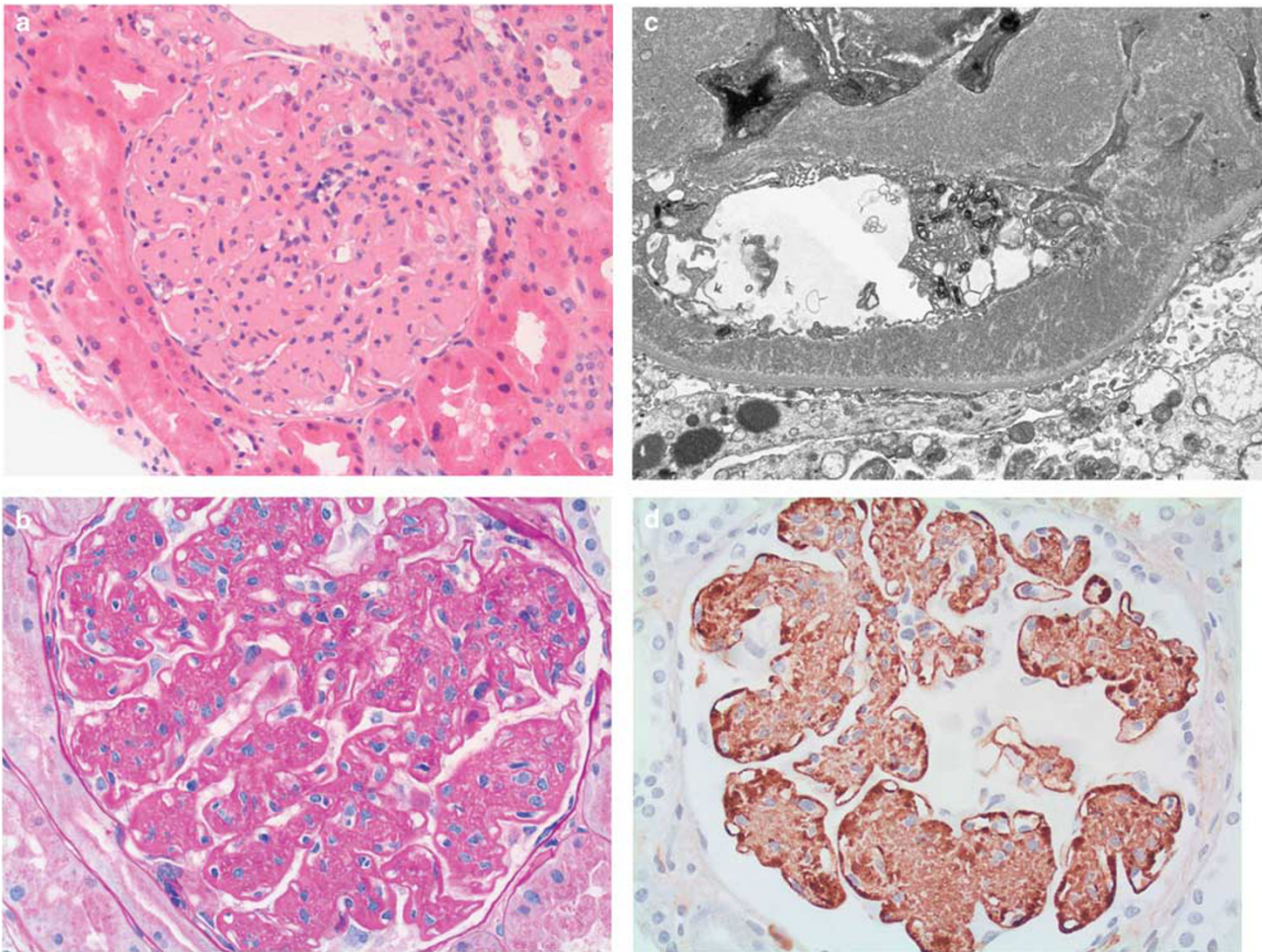
Figure 2 Recurrent fibronectin glomerulopathy in renal allograft of Patient 6. (**a**) Hematoxylin and eosin-stained tissue showing enlarged glomeruli and acellular expansion of mesangium ($\times 200$). (**b**) Mesangial deposits stain with periodic acid-schiff ($\times 400$). (**c**) Bulky deposits as seen on electron microscopic examination (uranyl acetate and lead citrate staining). (**d**) Immunoperoxidase staining for fibronectin with mouse monoclonal antibody to N-terminal of fibronectin shows strong global mesangial positivity ($\times 400$).
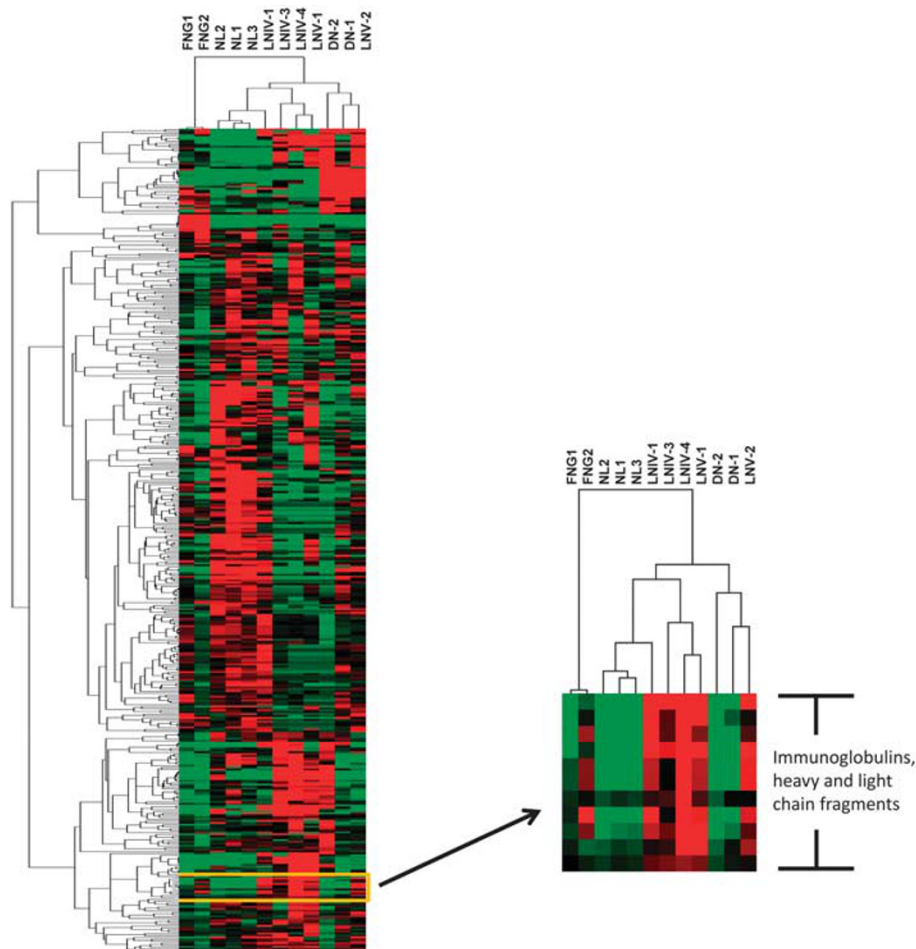
from patients with diabetic nephropathy and lupus nephritis.

A number of other proteins were altered in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. Unexpectedly, many components of the complement pathway were found in fibronectin glomerulopathy and diabetic nephropathy, diseases not associated with systemic complement activation or consumption. Glomeruli from biopsies with both class IV and class V lupus nephritis showed complement expression, as expected. In all the disease groups, podocyte-associated proteins and anti-oxidant proteins were markedly decreased relative to control. Several serum proteins were increased in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis compared with control, including clotting pathway components (fibrinogen), amyloid components, and apolipoprotein E.

To verify the laser capture microdissection/proteomic findings, kidney biopsies were stained for fibulin-1 and podocin, as representative proteins

that were increased or decreased, respectively, in the fibronectin glomerulopathy kidneys. Fibulin-1 showed mild diffuse staining in the expanded mesangium of patients with fibronectin glomerulopathy (Figure 4b). No fibulin-1 staining was seen in normal control kidneys (Figure 4a), in the Kimmelstiel–Wilson nodules of diabetic nephropathy (Figure 4c), or in the wire-loop lesions of lupus nephritis (Figure 4d). It is known that fibulin-1 is present in basement membranes,[31,32] consistent with the staining along the glomerular capillary walls seen in the glomeruli of diabetic nephropathy and lupus nephritis, and less intensely in normal kidneys.

Podocin is known to be expressed in the slit diaphragms of podocytes,[33] and podocin staining was seen in the podocytes along the glomerular capillary loops in normal glomeruli (Figure 5a). Staining was present but less intense in the biopsies with fibronectin glomerulopathy (Figure 5b) and lupus nephritis (Figure 5d). Staining was almost negative in the glomeruli with diabetic nephropathy (Figure 5c).

Figure 3 Heat map of glomerular proteins found in normal glomeruli, and glomeruli from patients with fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. Each vertical bar represents a single normal individual or patient, as indicated. Each horizontal bar represents a separate protein. Red indicates relatively more abundant proteins, and green less abundant proteins. The small inset map is a section representing immunoglobulin proteins and fragments, demonstrating their presence only in the five cases of lupus nephritis.

Given the unexpected similarities in protein expression from three clinically disparate types of glomerular disease, Ingenuity Pathway Analysis was used to functionally annotate glomerular proteins from normal and diseased kidneys (Figure 6). Representative biologic functions or canonical pathways based on the differentially expressed proteins are shown. The complement system, humoral immunity, and inflammatory response pathways were increased in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. Metabolic pathways appeared to be significantly down-regulated in all three glomerular diseases.

The discovery that fibulin was highly represented in the lesions of fibronectin glomerulopathy prompted us to determine if there were unique single-nucleotide polymorphisms in the fibulin-1 gene that segregated with fibronectin glomerulopathy. Although a number of single-nucleotide polymorphisms were found (data not

shown), none appeared to segregate with fibronectin glomerulopathy.

Discussion

The present work demonstrates the successful application of laser capture microdissection followed by mass spectrometry in the characterization of glomerular proteomes from clinical kidney biopsies. The high degree of clustering within the normal group and the fibronectin glomerulopathy group is an indication of the reproducibility of the method. There was more variability in the diabetic nephropathy and lupus nephritis patient groups; however, this is not unexpected. Kidney biopsies in diabetics are generally done in patients with atypical clinical courses, and in lupus nephritis there is a considerable spectrum of pathologic changes within individual lupus nephritis classes. Glomerular

Table 1 Glomerular protein expression in fibronectin glomerulopathy

Protein	Fibronectin glomerulopathy/N[a]	G[b]	Diabetic nephropathy/N	G	Lupus nephritis IV/N	G	Lupus nephritis V/N	G
Matrix proteins								
Fibronectin 1	14	665						
Fibronectin 3	14	663						
Fibronectin 8	14	668						
Fibronectin 14	14	66						
Fibulin-1	53	44						
Fibulin-1D	76	67						
Fibulin-5	16.6	13.8						
Laminin α-5	0.5	25						
Heparan sulfate proteoglycan core protein	2	19	3	35			1.5	5
Complement system								
C1q	13	7			20	12		
C3	4.4	33	37	39	10	109	13	120
C4B	4.2	15	7	19			10	44
C5	18	12	30	13	22	14	67	41
C8							13	5.5
C9					2.4	5.7	5	23.5
Factor H	10	5						
Serum proteins								
APOE	12	7	30	13			31	17
Amyloid P	4.6	10	9	19			4.8	8
Transthyretin	12	7						
Fibrinogen α-chain	26	39	47	46	35	49	45	54
Fibrinogen β-chain	40	32	62	30	56	41	67	41
Podocyte proteins								
Podocin	0.06	15	0.4	7	0.14	9.4	0.17	7
Synaptopodin	0.13	43	0.3	13	0.35	16.6		
A-actinin	0.7	14	0.7	8				
Complement receptor 1 isoform F precursor	0.06	15	0.1	7	0.07	15	0.09	9
Neprilysin	0.16	41	0.14	26	0.21	30	0.34	15
Tight junction protein ZO-1	0.24	22	0.09	24	0.2	22	0.27	14
MYH9	0.65	33						
Anti-oxidant proteins								
Peroxiredoxin-5	0.18	6						
Peroxiredoxin-6	0.07	12	0.12	6	0.08	9.8	0.10	7.5
Glutathione S-transferase A1	0.1	7			0.12	5.6		
Glutathione S-transferase A2	0.1	8			6	0.11		

[a]Ratio of normalized spectral counts of a protein found in a glomerular disease to the normalized spectral counts of the protein in control glomeruli.

[b]$G = G$-score, an estimate of significance if ≥ 5; if <5 spectral count ratio not given.

Table 2 Raw protein spectral counts for individual patients and controls

Controls			Fibronectin		Protein identification
1	2	3	1	2	
16	20	34	287	410	IPI:IPI00022418.1│FN1 isoform 1 of fibronectin
16	20	34	287	409	IPI:IPI00339223.1│FN1 isoform 3 of fibronectin
16	20	32	285	410	IPI:IPI00339228.1│FN1 isoform 8 of fibronectin
16	20	32	285	409	IPI:IPI00855777.1│FN1 isoform 14 of fibronectin
1	0	0	24	31	IPI:IPI00296534.2│FBLN1 isoform D of fibulin-1
0	0	0	18	19	IPI:IPI00889740.1│FBLN1 fibulin-1
0	0	0	7	5	IPI:IPI00294615.3│FBLN5 fibulin-5
48	51	61	33	19	IPI:IPI00783665.4│LAMA5 laminin subunit α-5
30	21	37	58	66	IPI:IPI00024284.5│HSPG2 basement membrane-specific heparan sulfate proteoglycan core protein
0	0	0	4	5	IPI:IPI00022394.2│C1QC complement C1q subcomponent subunit C
4	4	12	30	34	IPI:IPI00783987.2│C3 complement C3 (Fragment)
0	6	4	15	15	IPI:IPI00892604.1│C4B;C4A complement component C4B (Childo blood group) 2
0	0	0	7	1	IPI:IPI00816741.1│C5 complement component 5 variant (Fragment)
0	0	0	4	2	IPI:IPI00011261.2│C8G complement component C8 gamma chain
5	5	5	14	9	IPI:IPI00022395.1│C9 complement component C9
0	0	0	5	2	IPI:IPI00029739.5│CFH isoform 1 of complement factor H
0	0	0	7	2	IPI:IPI00021842.1│APOE apolipoprotein E
0	5	0	15	5	IPI:IPI00022391.1│APCS serum amyloid P-component
0	0	0	3	6	IPI:IPI00022432.1│TTR transthyretin
0	2	0	18	20	IPI:IPI00029717.1│FGA isoform 2 of fibrinogen α chain
0	0	0	10	19	IPI:IPI00298497.3│FGB fibrinogen β chain
8	10	5	0	0	IPI:IPI00014594.1│NPHS2 isoform 1 of podocin
34	20	28	7	0	IPI:IPI00027258.3│SYNPO Isoform 2 of synaptopodin
97	80	97	71	60	IPI:IPI00013808.1│TACTN4 α-actinin-4
7	0	2	0	0	IPI:IPI00787286.1│CR1 Complement component (3b/4b) receptor 1
35	20	27	7	3	IPI:IPI00247063.3│MME neprilysin
24	20	18	9	2	IPI:IPI00335824.5│TJP1 isoform short of tight junction protein ZO-1
187	183	244	172	116	IPI:IPI00019502.3│MYH9 isoform 1 of myosin-9
6	3	5	2	0	IPI:IPI00024915.2│PRDX5 isoform mitochondrial of peroxiredoxin-5; mitochondrial
8	4	7	1	0	IPI:IPI00220301.5│PRDX6 peroxiredoxin-6
6	0	6	0	0	IPI:IPI00657682.2│GSTA1 glutathione S-transferase A1
7	0	6	0	0	IPI:IPI00745233.2│GSTA2 glutathione S-transferase A2

immunohistochemistry was used to validate differential glomerular expression of selected proteins identified by the proteomic approach. Additional validation was provided by the finding that several of the differentially expressed proteins revealed by proteomic analysis are consistent with proteins known to be involved in glomerular injury, for example, complement activation products and immunoglobulins in lupus nephritis, or fibronectin in fibronectin glomerulopathy.

The most interesting findings from this study are the newly discovered changes in protein expression. This demonstrates that our approach is a robust platform that may inform disease pathogenesis. For example, despite the diversity of glomerular diseases examined, we found a number of glomerular protein changes that were common in the disease glomeruli but not the normal glomeruli, as follows:

(1) Several podocyte proteins, particularly those associated with the slit diaphragm, were significantly diminished in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. As all of these diseases are proteinuric, and because the attenuated podocyte proteins are important in the maintenance of the glomerular filtration barrier, decreased expression likely contributes to proteinuria. Interestingly, laser capture microdissection followed by microarray analysis of glomeruli from patients with focal segmental glomerulosclerosis showed a down-regulation of mature podocyte genes.[5] It is not presently known if the loss of these podocyte proteins and genes is a nonspecific response to podocyte injury and/or proteinuria, or is a primary event in the development of proteinuria. The latter scenario would imply a common pathogenic mechanism for different diseases.

(2) Complement components were found in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. The presence of complement proteins in the glomeruli of lupus nephritis patients is expected, though the patterns of class IV and class V are slightly different. Class IV lesions uniquely contained C1q, no C4 protein, and abundant C3 protein. The presence of C3 without C4 suggests significant alternative pathway activation, and is in agreement with our recent report of C3 consumption, but not C4 consumption, being independently associated with renal flare.[34] In contrast, class V lesions contained no C1q but detectable C4 protein, suggesting the involvement of the lectin pathway. Other complement components were also present in class V lesions, including abundant C9, likely reflecting the presence of the complement membrane attack complex as previously reported.[35]
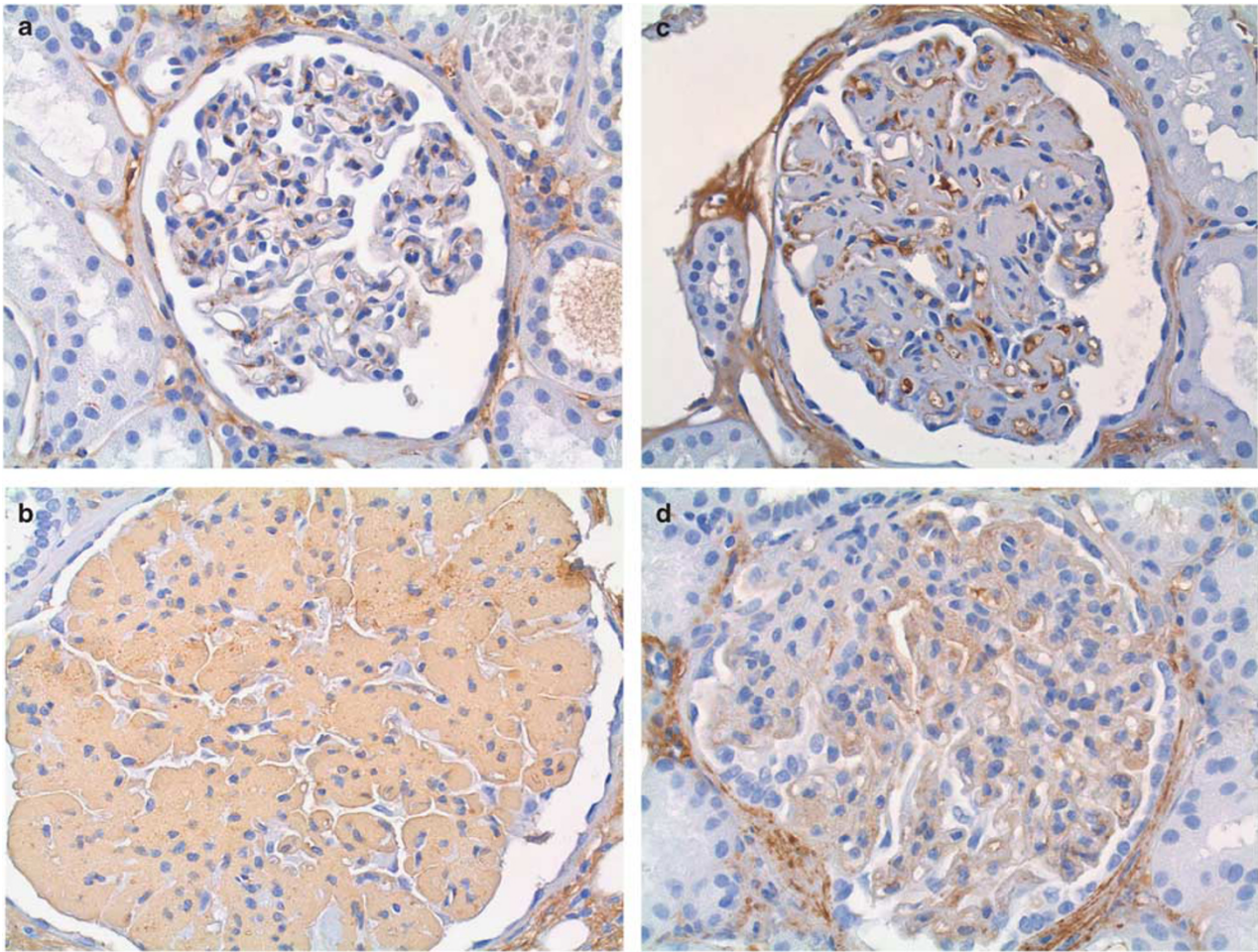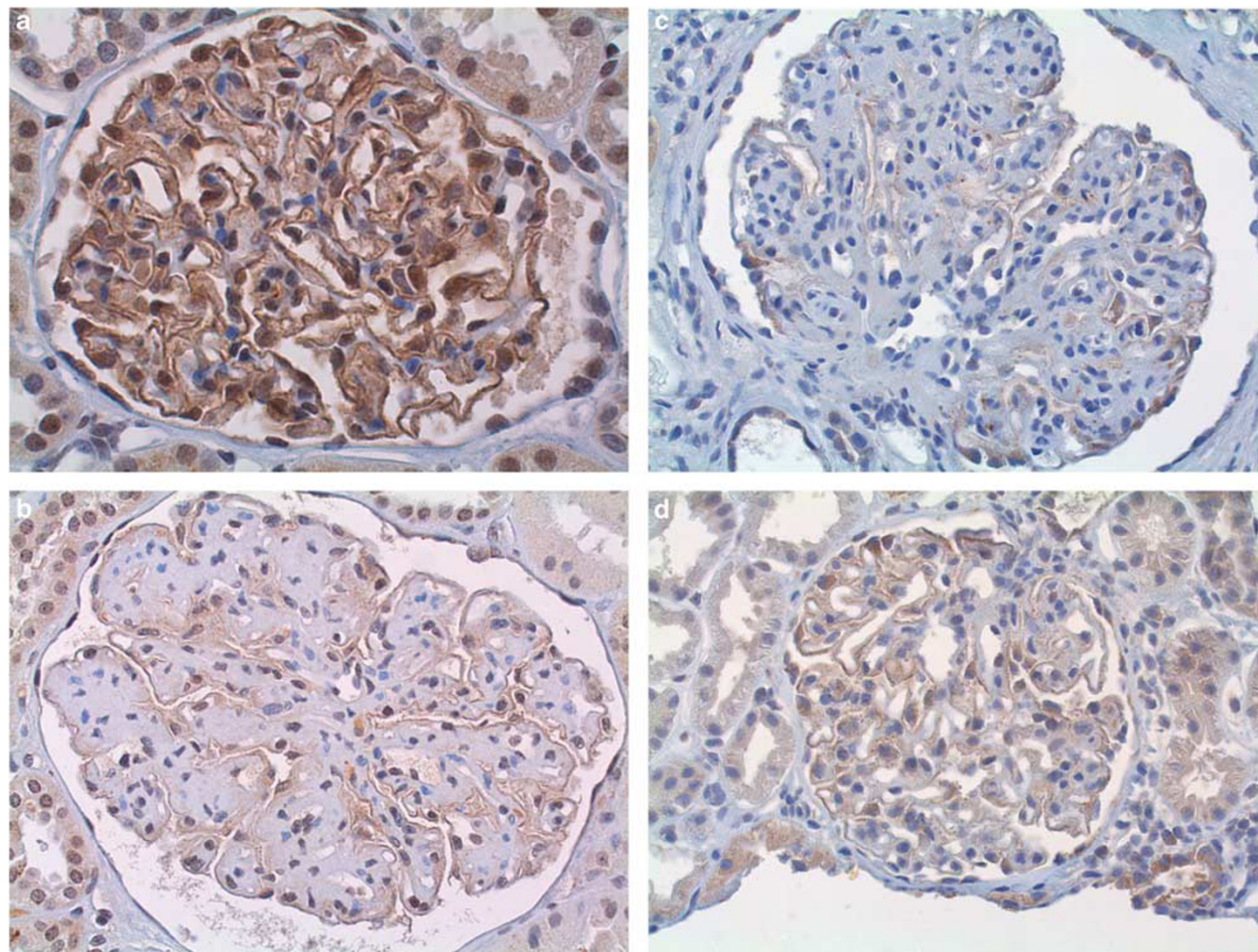
Figure 4 Immunoperoxidase staining for fibulin-1. (**a**) Baseline allograft biopsy with normal glomerulus shows no mesangial staining (× 400). (**b**) Biopsy with fibronectin glomerulopathy showing an enlarged glomerulus with mild but global mesangial staining (× 400). (**c**) Biopsy of patient with diabetic glomerulosclerosis. No staining in expanded mesangium. Staining along glomerular capillary wall is noted (× 400). (**d**) Biopsy of proliferative lupus nephritis with no staining in the mesangium.

In diabetic nephropathy, there are some data that complement may be involved in disease progression, possibly through the lectin-binding pathway, and C3 and the membrane attack complex (C5b-9) have been found in glomeruli (reviewed in Ostergaard *et al*[36]).

The presence of complement proteins in fibronectin glomerulopathy glomeruli is somewhat surprising, because immunohistochemical analysis of fibronectin glomerulopathy reveals inconsistent involvement of immunoglobulins or complement.[13] Fibronectin binds C1q via its collagenous tail,[37,38] which could explain the presence of C1q, but this binding does not result in complement activation.[39] Complement activation could occur through the alternative pathway, especially if the expression or function of complement regulators is compromised. Interestingly, a genome-wide linkage study identified a fibronectin glomerulopathy genetic susceptibility locus within the regulators of complement activation gene cluster.[40] However, no studies to date have identified abnormal expression or func-tion of regulators of complement activation gene cluster proteins in patients with fibronectin glomerulopathy.[41] Another possible explanation for the presence of complement proteins in fibronectin glomerulopathy is through glomerular complement expression. This has been demonstrated for C3 by *in situ* hybridization in glomeruli from lupus nephritis patients, but not from healthy controls.[42] Finally, this could simply represent nonspecific trapping of complement proteins, but if so this is significantly more than in normal glomeruli (see raw spectral counts, Table 2). The presence of complement proteins in the glomeruli of fibronectin glomerulopathy raises the possibility that complement-mediated damage may be involved in this nonimmune glomerular lesion. A recent proteomic evaluation of chronic allograft nephropathy also unexpectedly found complement pathway activation and reached a similar conclusion.[43] Limiting complement activation may therefore improve clinical outcomes in diseases not previously thought to involve clinically relevant complement activation.

Figure 5 Immunoperoxidase staining for podocin. (**a**) Baseline allograft biopsy with normal glomerulus shows staining of podocytes along capillary wall (× 400). (**b**) Biopsy with fibronectin glomerulopathy showing an enlarged glomerulus with weak staining in the capillary wall (× 400). (**c**) Biopsy of patient with diabetic glomerulosclerosis. No staining in podocytes (× 400). (**d**) Biopsy of proliferative lupus nephritis, with weak staining in podocytes (× 400).

(3) Anti-oxidant proteins were conspicuously under-represented in fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis glomeruli, but the proteomic approach cannot distinguish between a primary defect in anti-oxidant production, or a secondary loss of these proteins. Nonetheless, a reduction in glomerular anti-oxidants leaves the tissue open for oxidant injury.

(4) Pathway analysis based on the differentially expressed proteins suggested that compared with normal, metabolic pathways were suppressed in all three types of glomerular disease. This suggests that any type of glomerular injury may attenuate metabolism of glomerular cells, but more importantly, indicates that metabolomic evaluation of glomerular disease may uncover novel mechanisms of injury and repair.

To further demonstrate the utility of laser capture microdissection followed by glomerular proteomics, we studied fibronectin glomerulopathy in more detail. This uncommon glomerular disease results from the deposition of excessive matrix material in glomeruli. The hypothesis that fibronectin glomerulopathy is mechanistically related to abnormal glomerular accumulation of fibronectin is based mainly on evidence from immunohistochemical staining, which has limitations. Immunohistochemical staining can only test constituents for which specific antibodies are available, and can be falsely positive due to nonspecific trapping of antibodies, or falsely negative if antigens are blocked. By isolating glomeruli from kidney biopsies of affected patients, and doing unbiased proteomic analysis of the recovered glomerular proteins, we confirmed that fibronectin is the predominant protein in the lesions of fibronectin glomerulopathy glomeruli. In addition to several common pathways of glomerular injury activated in fibronectin glomerulopathy described above, we found considerable deposition of fibulin in these glomeruli. Among the diseases examined, fibulin accumulation appears to be unique to fibronectin glomerulopathy because it is not present in normal glomeruli, diabetic nephropathy, or lupus nephritis. Interestingly, fibronectin
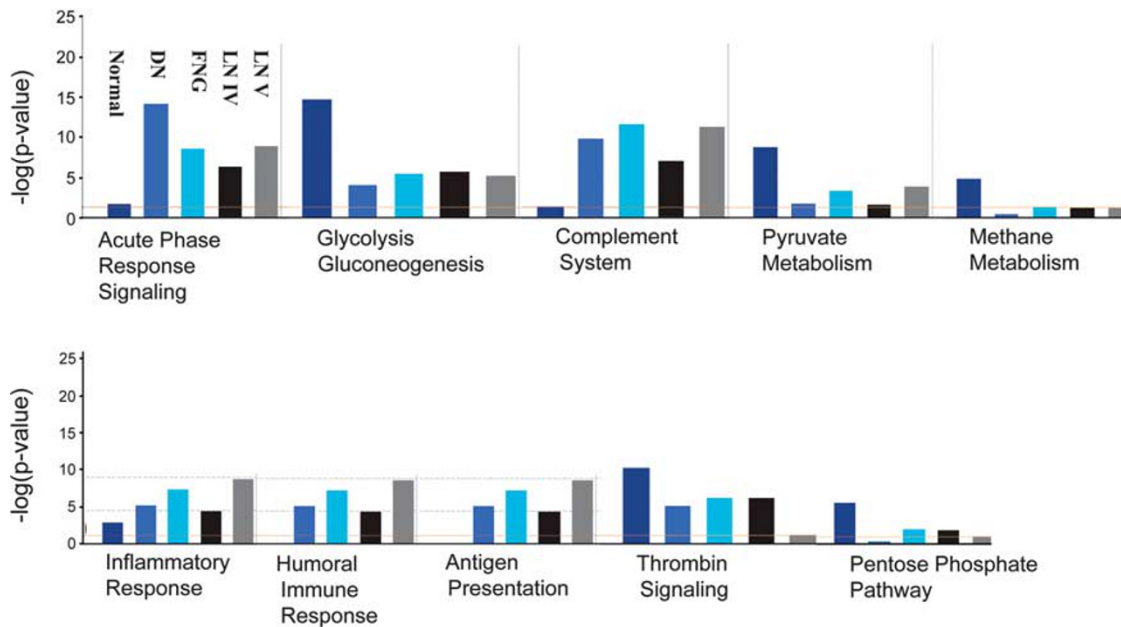
Figure 6 Representative biologic functions and pathways affected by fibronectin glomerulopathy, diabetic nephropathy, and lupus nephritis. Ingenuity Pathway Analysis was done based on differential protein expression in normal glomeruli, or glomeruli with fibronectin glomerulopathy, diabetic nephropathy, or lupus nephritis. The bars representing controls and the different disease states, as indicated, were assigned a probabilistic significance score shown as a *P*-value (*y* axis) for each pathway or function. The orange line represents the threshold for detection.

binds to fibulin-1 but not fibulin-5.[31,41,44,45] The fibulins appear to regulate several of the molecular activities of fibronectin. For example, fibronectin is important in cell–matrix interactions, and facilitates cell attachment, cell spreading, and cell migration.[46] Fibronectin also regulates the cytoskeleton, cellular morphology, and differentiation.[30] Fibulin-1 down-regulates fibronectin-facilitated cell spreading, adhesion, and motility.[47] These effects may occur because fibulin-1 inhibits fibronectin-mediated extracellular signal-regulated kinase activation.[47] Although fibulin-5 does not directly bind to fibronectin, it binds to the same $\beta1$ integrins as fibronectin ($\alpha5\beta1$, $\alpha4\beta1$), and like fibulin-1, blocks fibronectin-mediated cell spreading, motility, and proliferation.[48]

The role of fibulins plus fibronectin in the pathogenesis of glomerular injury in fibronectin glomerulopathy is not yet known; however, fibulins have been associated with other types of kidney disease. Specifically, fibulin-1 was identified as a candidate permeability factor in focal segmental glomerulosclerosis,[49] perhaps because it interferes with cell adhesion, and could therefore disrupt the podocyte-basement membrane relationship. Gene profiling of autosomal dominant polycystic kidney disease tissue showed markedly increased fibulin mRNA expression, and the protein was demonstrated in epithelial cells lining cysts.[50] Fibulin-1 has been shown to co-localize with ADAMTS-1 and increase its catalytic activity.[51] It is conceivable that in fibronectin glomerulopathy, the accumulated fibronectin helps localize fibulins to glomeruli, and

the fibulins inhibit cell spreading, adhesion, and motility, disrupting mesangial and/or podocyte function and facilitating the development of proteinuria.

In summary, the present study shows that the application of laser capture microdissection followed by liquid chromatography–tandem mass spectrometry analysis of kidney biopsies can provide large-scale quantitative proteomic profiles that may be important for characterizing glomerular diseases as well as determining mechanisms of disease pathogenesis. The ability to more clearly understand underlying molecular events critical to disease expression may open avenues for novel therapeutic interventions.

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Disclosure/conflict of interest

The authors declare no conflict of interest.

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