Semaphorin 3B-associated membranous nephropathy is a distinct type of disease predominantly present in pediatric patients

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Membranous nephropathy results from subepithelial antigen-antibody complex deposition along the glomerular basement membrane. Although PLA2R, THSD7A, and NELL-1 account for a majority (about 80%) of the target antigens, the target antigen in the remaining cases is not known. Using laser microdissection of PLA2R-negative glomeruli of patients with membranous nephropathy followed by mass spectrometry we identified a unique protein, Semaphorin 3B, in three cases. Mass spectrometry failed to detect Semaphorin-3B in 23 PLA2R-associated cases of membranous nephropathy and 88 controls. Semaphorin 3B in all three cases was localized to granular deposits along the glomerular basement membrane by immunohistochemistry. Next, an additional eight cases of Semaphorin 3B-associated membranous nephropathy were identified in three validation cohorts by immunofluorescence microscopy. In four of 11 cases, kidney biopsy also showed tubular basement membrane deposits of IgG on frozen sections. Confocal microscopy showed that both IgG and Semaphorin 3B co-localized to the glomerular basement membrane. Western blot analysis of five available sera showed reactivity to reduced Semaphorin 3B in four of four patients with active disease and no reactivity in one patient in clinical remission; there was also no reactivity in control sera. Eight of the 11 cases of Semaphorin 3B-associated membranous nephropathy were pediatric cases. Furthermore, in five cases, the disease started at or below the age of two. Thus, Semaphorin 3B-associated membranous nephropathy appears to be a distinct type of disease; more likely to be present in pediatric patients.

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Membranous nephropathy (MN) results from antibodies targeting an antigen in the glomerular basement membrane (GBM). MN is typically classified as primary MN, which has no identifiable underlying disease association, and secondary MN, where the MN may be associated with an autoimmune disease, infection, malignancy, and others.[1-3] The target antigen in primary MN has been identified as M-type phospholipase A_2 receptor (PLA2R), thrombospondin type-1 domain-containing 7A (THSD7A), and the recently described neural epidermal growth factor like-1 protein (NELL-1), respectively.[4-6] Exostosin 1/exostosin 2 (EXT1/EXT2) have also recently been identified as the putative antigen(s) in secondary (autoimmune) MN.[7] NELL-1 and EXT1/EXT2 were identified using laser microdissection of PLA2R-negative MN glomeruli followed by mass spectrometry. Using a similar approach, we sought to identify other novel antigen(s) in MN.

RESULTS

Discovery cohort

Laser dissection and tandem mass spectrometry detection of Semaphorin 3B in PLA2R-negative MN biopsies. We detected a unique protein Semaphorin 3B (Sema3B) by tandem mass spectrometry (MS/MS) in the glomeruli of 3 cases (patients 1-3) of MN (Figure 1). The counts ranged from 10 to 42 with an average total spectral count of 23.7 (SD, ±16.5). The average spectral counts of Sema3B were lower than PLA2R (86.1; SD,

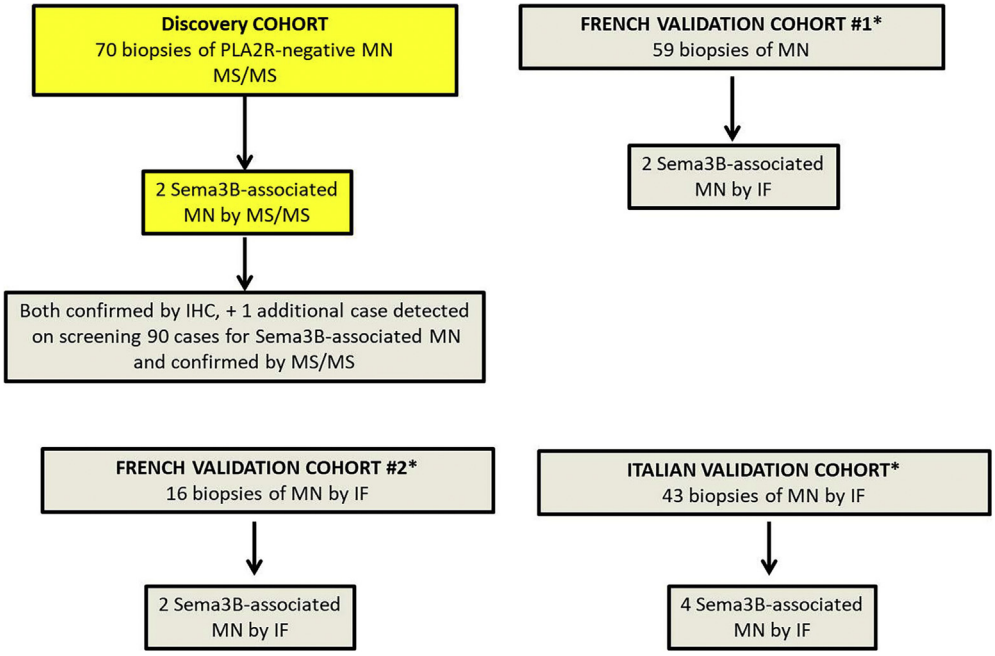

Figure 1 | Flowchart of the discovery and validation cohorts. Initial studies were done by mass spectrometry (MS) using 70 phospholipase A_2 receptor (PLA2R)–negative membranous nephropathy (MN). We detected Semaphorin 3B (Sema3B) in 2 cases that was then confirmed by immunohistochemistry (IHC). All 70 cases were negative for neural epidermal growth factor like-1 protein (NELL-1) and exostosin 1/exostosin 2 (EXT1/EXT2) by MS. We then studied an additional number (n = 90) of PLA2R-negative MN for Sema3B that yielded 1 positive Sema3B MN case. Only 4 cases were pediatric cases of the 160 cases of PLA2R-negative cases. Three validation cohorts were studied. The first French validation cohort included 59 adult cases of which 1 had a childhood onset (patient 5), and the second French cohort included 16 pediatric cases, and the Italian cohort included 43 pediatric cases. *All cases were negative for PLA2R, thrombospondin type-1 domain-containing 7A (THSD7A), NELL-1, and EXT1/EXT2 staining by immunofluorescence microscopy (IF). Yellow boxes indicate detection and confirmation of Sema3B by MS, and gray boxes indicate detection by IHC/IF.

±27.5), NELL-1 (63.1; SD, ±21.6), and EXT1/EXT2 (EXT1, 65.3 [SD, ±34.6]; EXT2, 83.4 [SD, ±38.4]) in PLA2R-, NELL-1–, and EXT1/EXT2-associated MN, respectively.[6,7] However, the presence of Sema3B was unique in this subset of MN, and importantly, all control cases including 15 time 0 kidney transplant biopsies, 73 other glomerulopathies, and 23 PLA2R-positive MN cases were negative for Sema3B. The spectral counts of Sema3B in the 3 cases, along with a representative sequence coverage map of Sema3B, are shown in Figure 2. The MS/MS match from 1 case is shown in Supplementary Figure S1. None of the cases showed any spectral counts for THSD7A or NELL-1. Subsequently, we screened over 2500 kidney biopsies, including pediatric cases, from our amyloid laboratory database (over 12,500 data files; both amyloid and nonamyloid cases) and did not find any kidney biopsy that showed any spectral counts for Sema3B. We included both adult and pediatric cases of C3 glomerulopathy. On analysis of other organs, we found trace amounts of Sema3B (1-3 spectral counts) in the heart and liver of 23 cases (over 2000 nonkidney cases were screened for Sema3B). All 4 classes of Igs were detected in Sema3B-associated MN, with average spectral counts of IgG1 25.0 (SD, ±4.0), IgG2 22.7 (SD, ±4.5), IgG3 25.0 (SD, ±9.5), and IgG4 17.0 (SD, ±5.3).

Immunohistochemical staining for Sema3B in PLA2R-negative MN biopsies. We performed immunohistochemical (IHC) staining for Sema3B in all 3 cases positive on MS/MS studies. All cases showed positive (2–3+/3) granular staining for Sema3B along the GBM. Importantly, there was no significant mesangial staining (Figure 3a). There was no staining along Bowman's capsule, tubular basement membranes, or in vessel walls. The positive Sema3B granular staining mirrored the granular IgG along the GBM seen in each case. All 45 control cases were negative for Sema3B, which included 3 pediatric cases of minimal change disease/focal segmental glomerulosclerosis. Representative negative staining for Sema3B in diabetes, focal segmental glomerulosclerosis, IgA nephropathy, minimal change disease, and PLA2R-associated MN is shown in Figure 3b.

Validation cohorts

Given the results obtained in the Mayo cohort showing 2 adults (patients 1 and 2) and 1 pediatric patient (patient 3) of Sema3B-associated MN, we analyzed 3 validation cohorts, 1 adult (French cohort 1) and 2 pediatric (Italian cohort, French cohort 2) cohorts (Figure 1). Sema3B was detected using immunofluorescence microscopy (IF) studies on paraffin sections after antigen retrieval. Patient 4 of the validation cohort had both IHC and IF studies performed.

French cohort 1 (Tenon Hospital). Two adult cases (patients 4 and 5) of 59 PLA2R-, THSD7A-, EXT1/EXT2-, and NELL-1–negative primary MN were positive for Sema3B. One case (patient 4) was detected by IHC at the Mayo Clinic and was then confirmed by IF studies on paraffin sections at Tenon

a

Probability Legend:
over 95%
80% to 94%
50% to 79%
20% to 49%
0% to 19%

Bio View:
1850 Proteins in 1674 Clusters
With 1849 Filtered Out

	Accession Number	Molecular Weight	Case 01	Case 02	Case 03
Semaphorin-3B OS=Homo sapiens OX=9606 GN=SEMA3B PE=2 SV=1	sp|Q13214|SEM3B_HUMAN	83 kDa	19	42	11
Immunoglobulin gamma-1 heavy chain OS=Homo sapiens OX=9606 PE=1 SV...	sp|P0DOX5|IGG1_HUMAN	49 kDa	25	29	21
Immunoglobulin heavy constant gamma 2 OS=Homo sapiens OX=9606 GN=I...	sp|P01859|IGHG2_HUMAN	36 kDa	24	18	28
Immunoglobulin heavy constant gamma 3 OS=Homo sapiens OX=9606 GN=I...	sp|P01860|IGHG3_HUMAN	41 kDa	20	19	36
Immunoglobulin heavy constant gamma 4 OS=Homo sapiens OX=9606 GN=I...	sp|P01861|IGHG4_HUMAN	36 kDa	20	11	21
Secretory phospholipase A2 receptor OS=Homo sapiens OX=9606 GN=PLA2R1 ...	sp|Q13018|PLA2R_HUMAN	169 kDa	2	5	(0)

b

sp|Q13214|SEM3B_HUMAN (100%), 83,121.5 Da
Semaphorin-3B OS=Homo sapiens OX=9606 GN=SEMA3B PE=2 SV=1
31 exclusive unique peptides, 37 exclusive unique spectra, 45 total spectra, 415/749 amino acids (55% coverage)

Figure 2 | Proteomic identification of Semaphorin 3B (Sema3B) in phospholipase A_2 receptor (PLA2R)–negative membranous nephropathy (MN). Glomeruli were microdissected and analyzed using mass spectrometry (MS) as described in the Methods. (**a**) Detection of Sema3B in 3 cases of PLA2R-negative MN. Numbers in green and yellow boxes represent spectral counts of MS/MS matches to a respective protein. Top panel shows spectral counts of Sema3B in the 3 cases. Panels 2–4 show IgG1, IgG2, IgG3, and IgG4, and bottom panel shows baseline spectral counts of PLA2R. (**b**) Representative sequence coverage map of Sema3B from 1 case. Amino acids highlighted in bold letters over yellow background are the amino acids detected. Note the extensive coverage. Green highlighted boxes indicated amino acids with artefactual chemical modification induced by mass spectrometry, such as oxidation of methionine.

Hospital (Figure 4a and b). Patient 5 had 3 biopsies performed at the age of 1, 6, and 19 years, showing granular staining for Sema3B along the GBM (biopsy at age 1 and 19 shown in Figure 4c and d and Supplementary Figure S3E and F).

It became clear that Sema3B-associated MN was enriched in pediatric patients. After this unique finding, we then screened for Sema3B in a larger cohort of MN in the pediatric age group (Italian cohort and French cohort 2).

Italian cohort (Bambino Gesù Pediatric Hospital, Rome). Four cases (patients 6, 7, 8, and 9) of 43 pediatric cases negative for PLA2R, THSD7A, EXT1/EXT2, and NELL-1 showed bright granular staining for Sema3B along the GBM (Figure 4e–h). Of note, patients 7 and 8 were siblings. Of the 43 cases, 6 had lupus class V MN, including 3 with class III + V, and were all negative for Sema3B.

French cohort 2 (Trouseau Pediatric Hospital, Paris). An additional 2 cases (patients 10 and 11) of 16 pediatric cases negative for PLA2R, THSD7A, EXT1/EXT2, and NELL-1 showed the bright granular deposits of Sema3B along the GBM (Figure 4i and j). Of the 16 cases, 9 had lupus class V MN and were all negative for Sema3B.

Figure 4 shows the granular GBM staining and absence of mesangial or Bowman capsule staining in all cases. An adult and pediatric case of Sema3B-negative MN is also shown (Figure 4k and l).

Confocal microscopy

We performed confocal IF to determine whether Sema3B and IgG co-localized along the GBM (Figure 5). Bright granular staining for Sema3B (green) and IgG (red) was seen along the GBM (Figure 5a and b). Furthermore, superimposition of the 2 signals showed co-localization resulting in a yellow signal (Figures 5c). Laser quantitative analysis (Figure 5d and e) also confirmed the co-localization of Sema3B and IgG, further corroborating that the subepithelial deposits contained both Sema3B and IgG. A second case (patient 6, 2 years old) is shown in Supplementary Figure S2.

Western blot analysis

Western blot analyses in nonreducing and reducing conditions were performed using human Sema3B full-length recombinant proteins to determine the presence of circulating anti-Sema3B antibodies in the available serum of 5 patients (patients 3, 4, 5, 8, and 11) (Figure 6). Four of the 5 patients showed reactivity against Sema3B under reducing but not under nonreducing conditions. A band was seen at around 81 kDa representing the full-length protein. Serial serum samples of patient 4, who was positive for anti-Sema3B antibody, were also available for time-course analysis of antibody levels. Results show progressive decrease with disappearance of antibodies after rituximab treatment. Patient 5, who was sampled after a long-term immunosuppressive treatment, was negative by Western blot analysis. In contrast, sera from patients with proteinuric conditions including PLA2R-, THSD7A-, and NELL-1–associated MN; IgA nephropathy; focal segmental glomerulosclerosis; and minimal change disease and control subjects did not show reactivity with Sema3B when analyzed under identical conditions.

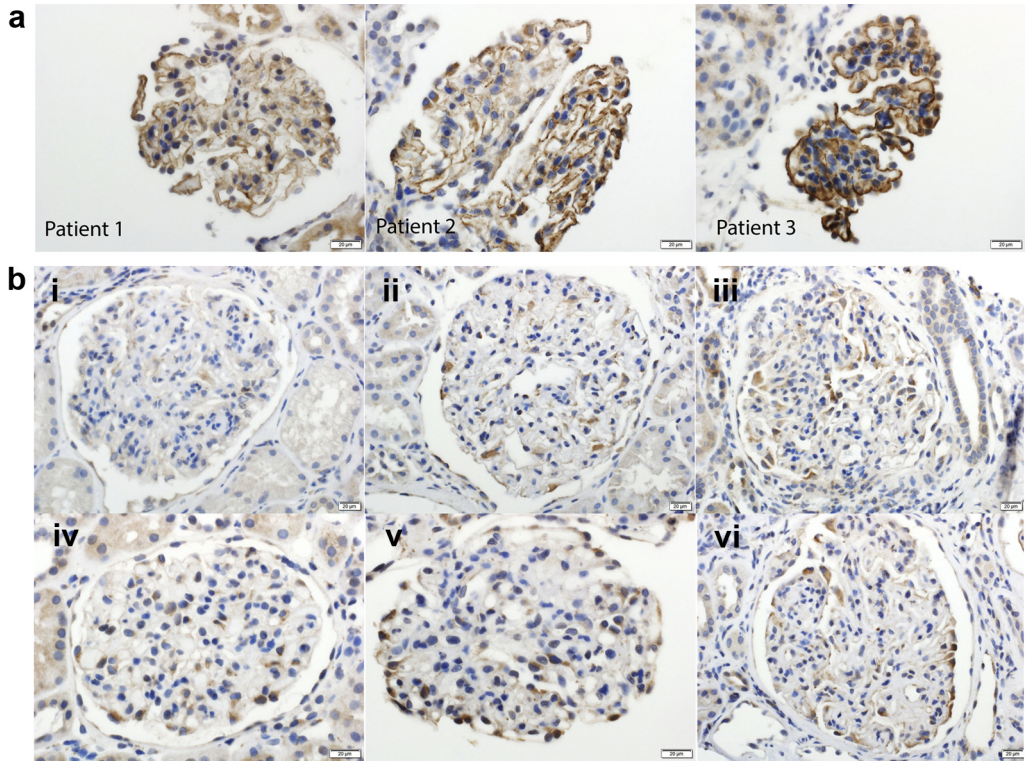

Figure 3 | Immunohistochemical stain for Semaphorin 3B (Sema3B) in Sema3B-associated membranous nephropathy (MN) (Mayo Clinic cohort), phospholipase A_2 receptor (PLA2R)–associated MN, and control cases. (**a**) Bright granular capillary wall staining for Sema3B along the glomerular basement membranes in 3 cases of Sema3B-associated MN. (**b**) Negative Sema3B staining in a representative case of (**i**) diabetic glomerulosclerosis, (**ii**) focal segmental glomerulosclerosis, (**iii**) IgA nephropathy, (**iv**) minimal change disease, (**v**) time zero transplant biopsy, and (**vi**) PLA2R-positive MN. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

Clinical and kidney biopsy findings of Sema3B-associated MN

We identified 11 cases of Sema3B-associated MN, 3 from the Mayo Clinic cohort, 2 from the French validation cohort 1, 4 from the Italian validation cohort, and 2 from the French validation cohort 2 (Table 1). There were 7 male and 4 female patients. Of the 11 cases, 8 (73%) were pediatric patients (<18 years of age) and 3 (27%) were adult patients. Of the Mayo Clinic cohort (patients 1–3), 1 was a pediatric patient (1 of 4 pediatric PLA2R-negative MN cases tested for Sema3B, 25%) and 2 were adults (2 of remaining 156 primary and secondary PLA2R-negative MN including class V membranous lupus nephritis tested, 1.28%). Of the validation cohorts (Paris and Rome combined), there were 8 pediatric patients (8 of 60 PLA2R-negative pediatric MN tested including case 5, 13.3%) and 1 adult patient (1 of 58 PLA2R-negative adult MN tested excluding patient 5, 1.7%). In patient 5, MN was diagnosed at the age of 1 year (biopsy performed because of steroid-resistant nephrotic syndrome), and repeat biopsies performed 6 and 18 years later showed grade II MN. Each of the pediatric cohorts included 9 patients with lupus MN class V. In total, 6 of 41 (14.6%) nonlupus pediatric cases tested were Sema3B positive.

The average age of pediatric patients at disease onset was 6.9 years (SD, ±6.8), and the average age of the 3 adult patients was 36.3 years (SD, ±7.2). Two children had extrarenal features of autoimmunity (type 1 diabetes in patient 10 and idiopathic thrombocytopenic purpura with positive anti-nuclear antibody in patient 11). The average serum creatinine was 0.5 mg/dl (SD, ±0.2), and proteinuria was 7.4 g/24 h (n = 7; SD, ±5.7).

Kidney biopsy in all cases showed features of MN with thickened GBMs. Immunofluorescence microscopy showed granular IgG (Table 2) and Sema3B deposits along the glomerular capillary walls. Interestingly, 4 cases (patients 3, 5, 6, and 9, all pediatric, <2 years of age at onset) also showed tubular basement membrane staining for IgG in a granular pattern (Figure 7, Supplementary Figure S3A and B). In patient 5, granular IgG deposits along tubular basement membranes were noted in the first biopsy (at 1 year of age) but disappeared thereafter. However, the tubular basement membrane deposits were negative for Sema3B staining in all biopsies (Figure 7, Supplementary Figure S3D and E). Complete IgG subtyping done in 4 cases (3 in the Mayo Clinic cohort and 1 in French cohort 1) showed IgG1 in 3 cases and IgG1 and IgG4 in 1 case. IgG4 staining only was done in 4 cases of the Italian cohort, and all 4 cases were negative for IgG4 (Supplemental Table S1). Electron microscopy was performed in all patients except patients 4 and 11. It showed glomerular subepithelial deposits in all cases that were graded as stage II in most cases. The deposits seen along tubular

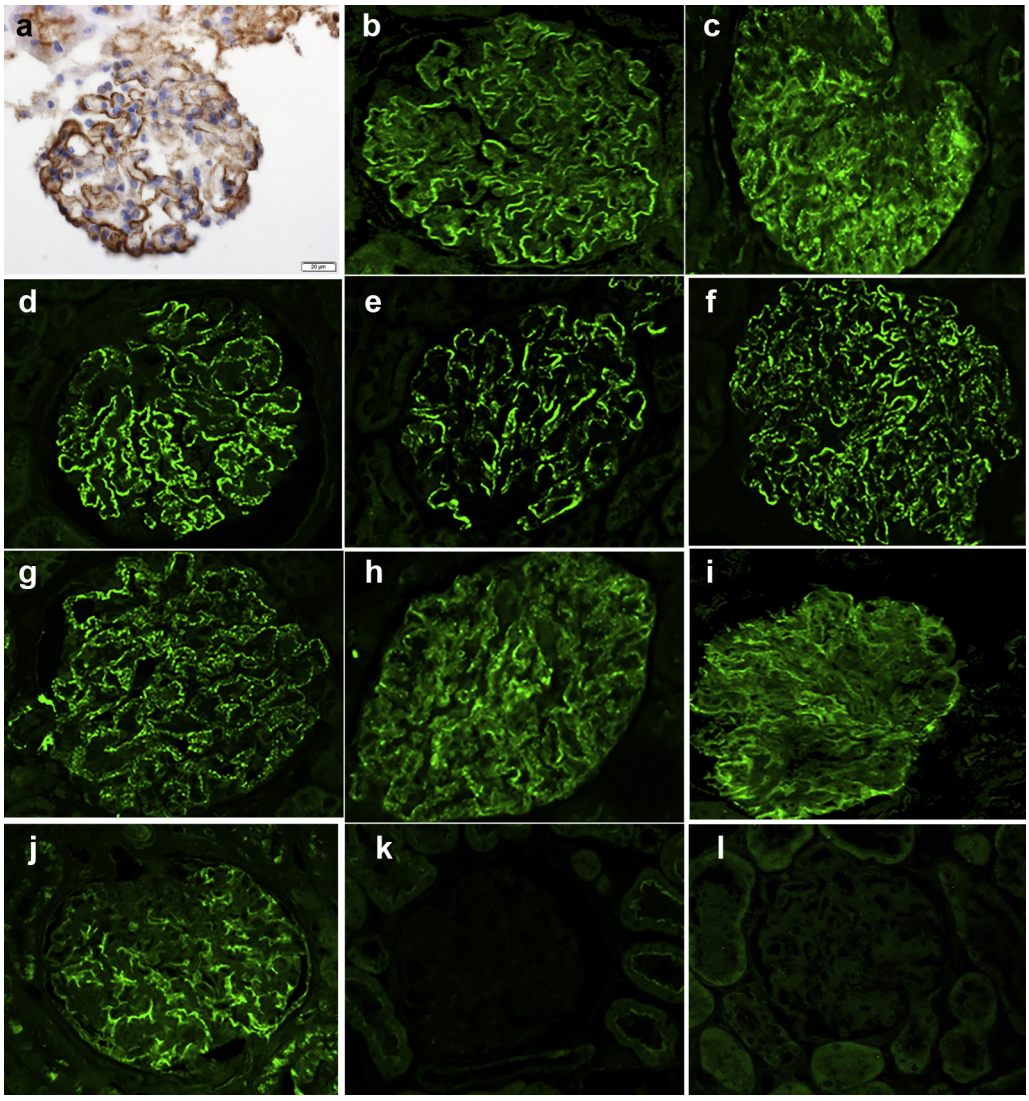

Figure 4 | Immunofluorescence microscopy for Semaphorin 3B (Sema3B) in the validation cohorts. (**a,b**) Patient 4 stained by immunohistochemistry and immunofluorescence microscopy. (**c,d**) Patient 5, with biopsies at ages 1 and 19. (**e–j**) Patients 6, 7, 8, 9, 10, and 11. Patients 6 to 11 are pediatric patients. (**k**) An adult PLA2R-positive membranous nephropathy control case. (**l**) Pediatric PLA2R-negative membranous nephropathy control case. To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

basement membranes were confirmed or revealed by electron microscopy in 3 cases (patients 3, 6, and 9). Tubuloreticular inclusions were noted in these cases as well.

Treatment and follow up

Mayo Clinic cohort. Patients 1 and 2 received only angiotensin-converting enzyme inhibitors and went into remission with minimal proteinuria (<500 mg/24 hours) at last follow-up of 18 and 24 months, respectively. Patient 3 (Figures 6 and 7) received steroids, tacrolimus, and angiotensin-converting enzyme inhibitors and also went into remission. At last follow-up (24 months) the proteinuria was 150 mg/24 hours.

French cohort 1 (adult). Patient 4 was treated for 4 months with angiotensin-converting enzyme inhibitors. Because of persisting severe nephrotic syndrome, she was given rituximab, 2 infusions of 375 mg/m^2 at 2-week interval, followed by a third infusion at 3 months. Thereafter, she reached complete remission, which is maintained after 10 years (proteinuria < 500 mg/24 h, no detectable anti-Sema3B antibody in serum; Figure 6).

Patient 5 was diagnosed at the age of 1 year (Supplementary Figure S3B and E). He failed to respond to cyclosporine, and from age 5 to 7 years he was treated with mycophenolate mofetil that induced complete remission. Six months after mycophenolate mofetil withdrawal, a relapse occurred. A second biopsy showed MN stage 2 with disappearance of granular tubular deposits. Mycophenolate mofetil (500 mg twice daily) was resumed and increased to 1 g twice daily. Ten years later because of the reappearance of isolated proteinuria without nephrotic syndrome, the patient was referred at the age of 19 years to discuss immunosuppressant

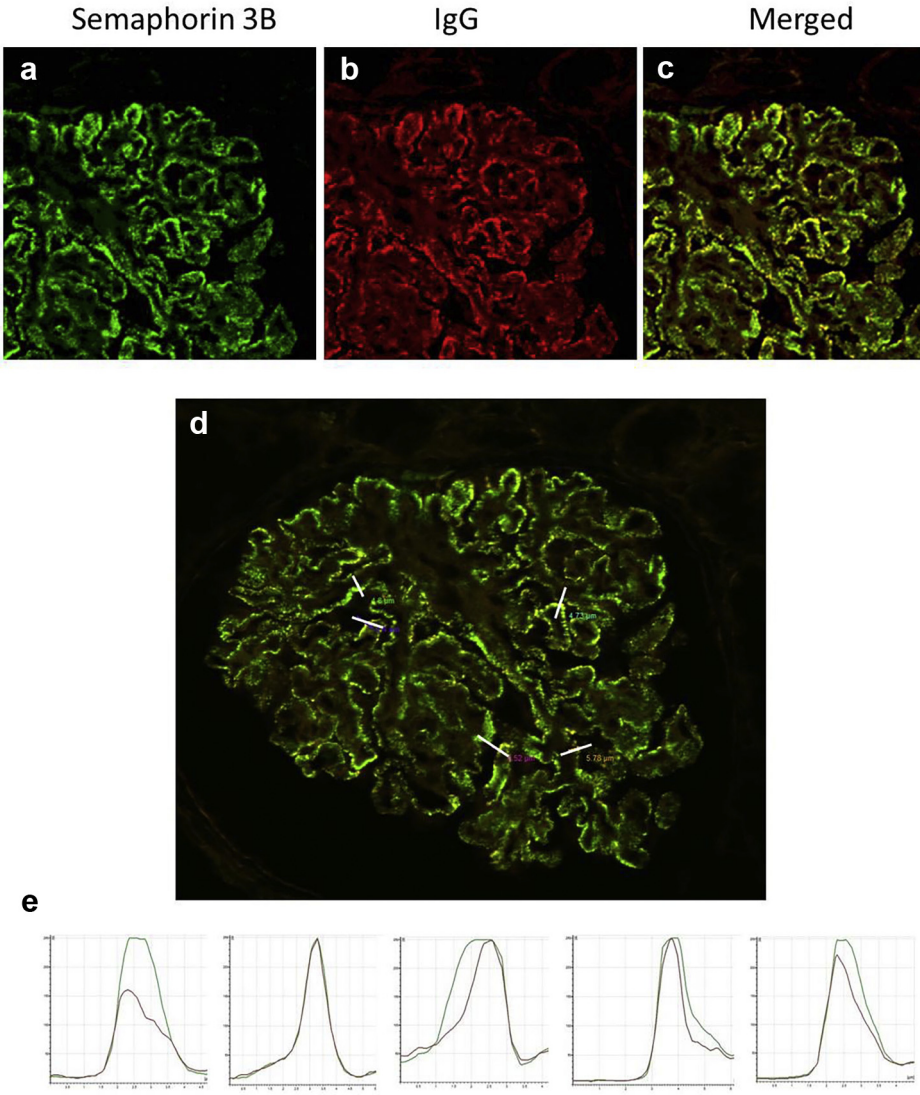

Figure 5 | Detection of Semaphorin 3B (Sema3B) and IgG in glomerular immune deposits in Sema3B-associated membranous nephropathy by confocal immunofluorescence microscopy analysis. Glomeruli were double-labeled with anti-Sema3B (**a**, green) and anti-human IgG (**b**, red), with the right panel (**c**) showing the merged image. (**d**) Enlarged image of the merged image. The white lines show the places where fluorescence was recorded across sections of a representative capillary loop. (**e**) Quantitative analysis of the fluorescence recorded across sections of representative capillary loops. Note the superimposition of the 2 signals, which indicates that subepithelial immune deposits contain Sema3B (green) and IgG (red). The confocal microscopy studies shown are from the biopsy of patient 5 (biopsy done at the age of 19 years). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

withdrawal. Because of persisting low-level proteinuria (400 mg/24 h) with normal renal function (serum creatinine, 0.9 mg/dl), a third kidney biopsy (Figure 5 and Supplementary S3C and F) was performed. The kidney biopsy showed MN stage 2 with abundant deposits by IF (IgG1, C3, Sema3B) and electron microscopy (subepithelial electron-dense deposits). Mycophenolate mofetil was then tapered. One year later the patient is still in complete remission. Anti-Sema3B antibodies were not detected (Figure 6).

Italian pediatric cohort. Patient 6 (onset $<$ 2 years of age with nephrotic-range proteinuria) was biopsied at disease onset (Supplementary Figures S2 and S3A and D) because her father had MN. She received oral prednisone tapered in 6 months and cyclosporine. This led to a complete remission, with a follow-up of 13 months.

Patients 7 and 8 are siblings. Patient 7 presented at age 17 years with proteinuria of 16 g/24 h and was treated with oral prednisone monotherapy. Because of a partial response after 2 months, a renal biopsy was done that showed MN. The patient was started on an angiotensin-converting enzyme inhibitor and cyclosporine. Prednisone was tapered and discontinued at the end of 4 months. The patient went into remission and has no proteinuria at 19-month follow-up.

Patient 8 presented at age 2 years with nephrotic-range proteinuria, normal renal function, and negative anti-

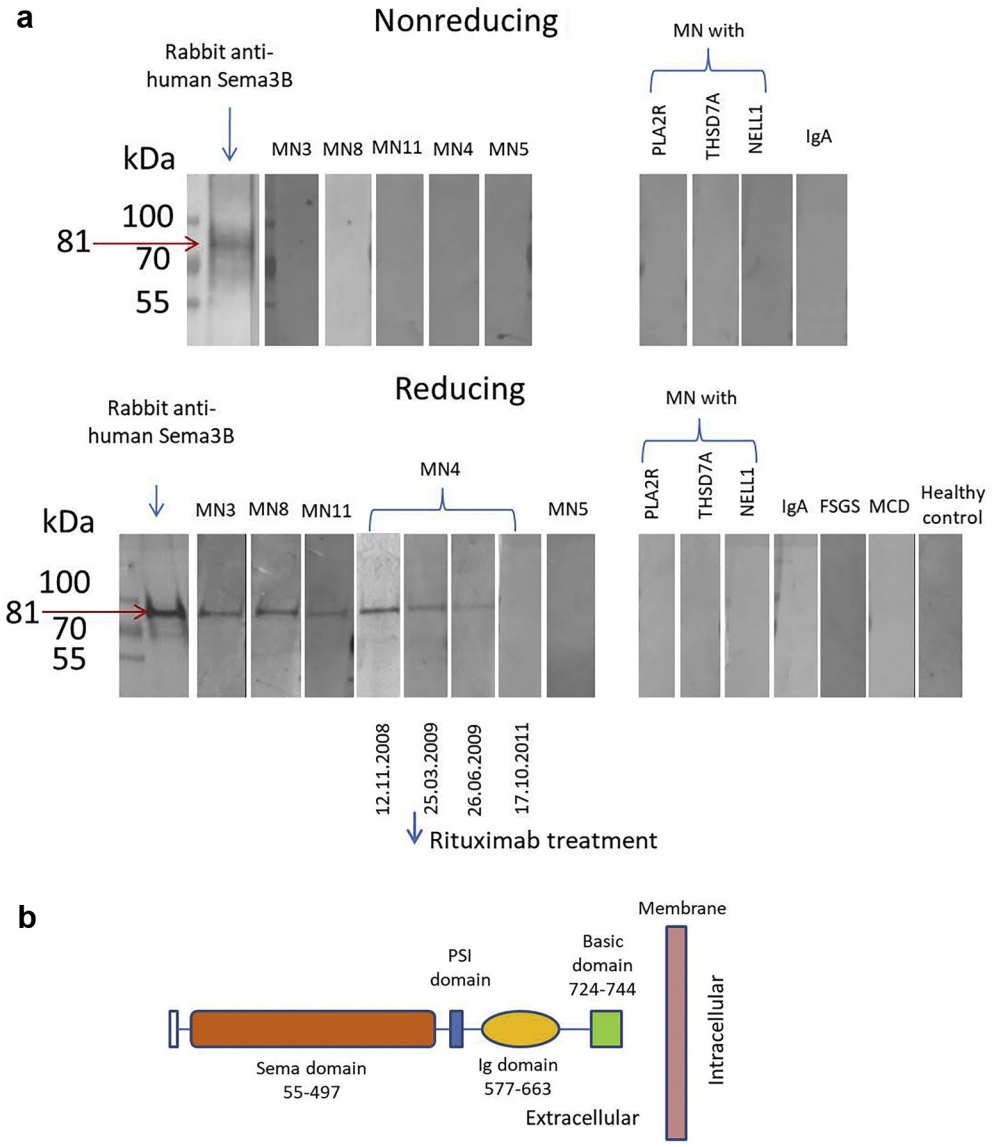

Figure 6 | Detection of anti-Semaphorin 3B (Sema3B) antibodies in the serum by Western blot analysis. (**a**) Reactivity of serum samples with recombinant Sema3B protein. Under nonreducing conditions Sema3B is detected by control anti-Sema3B rabbit antibody as a band at around 80 kDa. This band is not detected by patient's sera and control sera. Under reducing conditions Sema3B is detected as a sharp band at around 80 kDa by the control anti-Sema3B antibody. In this condition, the same band is also detected by patient's sera but not by sera from patients with other kidney diseases or healthy control subjects. In patient 4, samples were available at the indicated time points. Treatment with rituximab was started on March 18, 2009. Note the disappearance of the band afterward. Patient 5 was sampled after long-term immunosuppressive treatment. Patients 3, 4, 5, 8, and 11 are labeled as MN3, MN4, MN5, MN8, and MN11, respectively. (**b**) Molecular structure of Sema3B, with large sema domain region, plexin-semaphorin-integrin (PSI) domain, Ig domain, and short C terminal basic domain.

nuclear antibody. Kidney biopsy showed MN. Because the biopsy showed a full-house (IgG3+, IgM 2+, IgA 3+, C3 2+, C1q 3+) pattern of staining on IF, the patient was treated with i.v. cyclophosphamide and methylprednisolone bolus followed by maintenance low-dose prednisone and azathioprine. Treatment was tapered and discontinued after 2 years. The child experienced a first relapse (urinary protein-to-urinary creatinine ratio $\geq$ 1) 2 years later that showed MN on kidney biopsy that responded to cyclosporine. The patient had a relapse again at 9 years of age, and another repeat kidney biopsy (the one examined in this study) confirmed MN. He was treated with i.v. rituximab with relapse on tapering of cyclosporine despite repeated rituximab doses and therefore was changed to tacrolimus, with full response. At last follow-up (14 years from onset) he was in remission with normal renal function on low-dose tacrolimus and angiotensin-converting enzyme inhibitor.

Patient 9 presented with nephrotic-range proteinuria (urinary protein-to-urinary creatinine ratio of 16.75) at 2 years of age and was treated with oral prednisone with partial response after 4 weeks of treatment. A renal biopsy was performed that showed MN. The patient went into

Table 1 | Clinical findings in Semaphorin 3B–associated membranous nephropathy

Case	Age (yr)/sex	Urinary protein (g/24)	Serum creatinine (mg/dl)	Spontaneous remission vs. immunosuppressive treatment	Serum creatinine/urinary protein/24 h (follow-up)
1	41/F	7.9	0.74	Spontaneous	0.6/no proteinuria (16 mo)
2	28/F	6.2	0.4	Spontaneous	0.43/400 mg (18 mo)
3	2/M	5	0.5	Immunosuppressive	0.35/150 mg (24 mo)
4	40/F	17.3	0.9	Immunosuppressive	0.6/no proteinuria (10 yr)
5	19/M[a] (onset 1 yr)	0.4	0.7	Immunosuppressive	0.9/400 mg (18 yr)
6	1 yr and 9 mo/F	UP/CR ratio 6.81, microhematuria ++	0.21	Immunosuppressive	UP/CR ratio 0.23 (13 mo)
7	17/M	UP/CR ratio 0.78	0.6	Immunosuppressive	UP/CR ratio 0.1 (19 mo)
8	9/M (onset 2 yr)	UP/CR ratio 0.94	0.45	Immunosuppressive	UP/CR ratio 0.09 (14 yr)
9	2/M	UP/CR ratio 1.95	0.13	Immunosuppressive	UP/CR ratio 0.12 (5 yr)
10	14/M	3	0.64	Lost to follow-up	n/a
11	16/M	12	0.83	Immunosuppressive	Dialysis

n/a, not available; UP/CR, urinary protein/urinary creatinine.
[a]Membranous nephropathy was diagnosed at the age of 1 year (biopsy performed because of steroid-resistant nephrotic syndrome), repeat biopsy in 2006 and 2019 at age 19 years.

complete remission after treatment with cyclosporine and oral prednisone. However, each time cyclosporine was tapered proteinuria reappeared, and at age 7 years he was treated with rituximab. At last follow-up he was in complete remission.

French cohort 2 (pediatric). Patient 10 was lost to follow-up. Patient 11 (Figure 6) progressed to end-stage kidney disease within 3 years, being unresponsive to rituximab, mycophenolate mofetil, and cyclosporine.

DISCUSSION

MN is the most common cause of nephrotic syndrome in white adults. On the other hand, it is a rare cause of nephrotic syndrome in children. In the last decade, groundbreaking research has led to the identification of target MN antigens including PLA2R, THSD7A, NELL-1, and putative antigens EXT1/EXT2.[4–7] However, these antigens do not account for all cases of MN, and in a significant number of MN the target antigen is still elusive. Using a combination of laser microdissection, MS/MS, and IHC techniques we studied a subgroup of PLA2R-negative MN to determine whether we could identify novel target antigens in this remaining group of MN.

Our previous MS/MS studies confirmed high spectral counts of NELL-1 and EXT1/EXT2 in a cohort of PLA2R-negative MN.[6,7] Using the same cohort, we then searched for other proteins that were unique to this group of PLA2R-negative MN. The criteria used to identify a putative antigen/protein from over 1500 to 2000 proteins detected on MS/MS were the identification of a unique protein and the absence (or low baseline spectral counts) of the protein in control cases and other cases of MN including both PLA2R-positive and -negative MN. Using this approach, we found moderate spectral counts of a unique protein, Sema3B, in 3 cases of MN. We confirmed the MS/MS finding by IHC and further validated our findings in 2 French cohorts and an Italian cohort.

Eight (72.7%) of 11 Sema3B-associated MN were pediatric patients, of which 5 patients developed MN on or before the age of 2 years. The remaining 3 patients developed MN at the ages of 14, 16, and 17 years. Of the 3 adult cases identified, the average age was 36.3 years, which is still significantly lower than the primary MN age group. Thus, Sema3B-associated MN appears primarily to involve pediatric patients and young adults.

Table 2 | Kidney biopsy findings in Semaphorin 3B–associated membranous nephropathy

Case	Sclerosed/total glomeruli	IFTA	Immunofluorescence microscopy	Electron microscopy	Tubular basement membrane deposits
1	1/22	0	IgG 3+ C3 2+	I–II	No
2	1/36	0	IgG 3+ C3 2+	I–II	No
3	0/60	10	IgG 3+ C3 3+	I–II	Yes
4	0/19	0	IgG 1+ C3 1+ C1q 1+	Not done	Not done
5	0/6	0	IgG 3+ C3 2+	II	Yes
6	0/20	0	IgG 3+ C3 2+ C1q 1+	III	Yes
7	1/30	0	IgG 3+ C3 3+ C1q 2+	II	No
8	3/16	30	IgG 3+ C3 2+	II	No
9	1/18	0	IgG 3+ IgM 1+ C3 +	II	Yes
10	0/9	0	IgG 3+ IgA 2+ C3 3+ C1q 1+	II	No
11	0/9	5	IgG 3+ IgM 3+ C1q2+	Not done	Not done

IFTA, interstitial fibrosis and tubular atrophy.

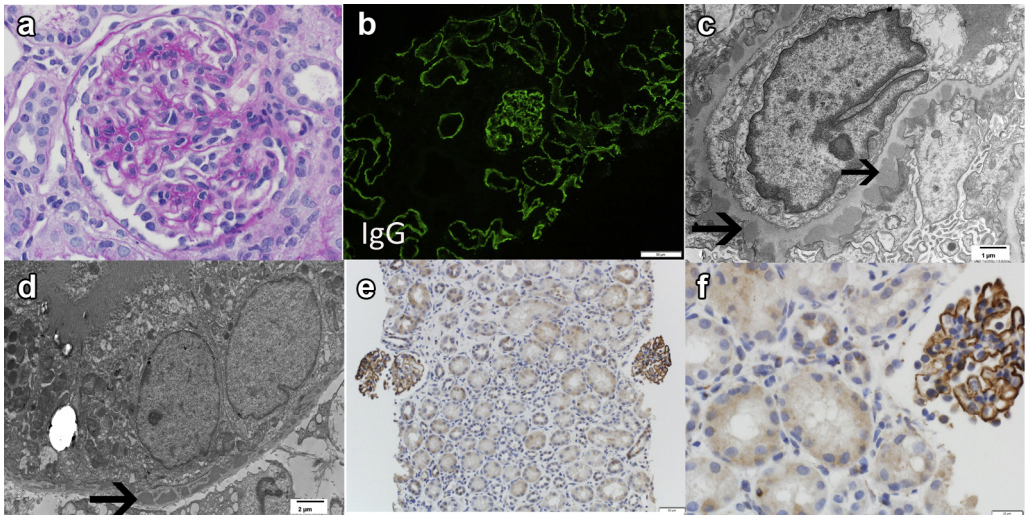

Figure 7 | Representative kidney biopsy findings (patient 3). (**a**) Light microscopy showing slightly thickened glomerular capillary walls (original magnification ×40). (**b**) Immunofluorescence microscopy showing glomerular and tubular basement membrane staining for IgG. (**c,d**) Electron microscopy showing (**c**) subepithelial and (**d**) tubular basement membrane deposits. (**e**) Anti-Semaphorin 3B (Sema3B) staining along the glomerular basement membrane. (**f**) Higher power showing that the tubular basement membranes are negative for Sema3B (original magnification ×60). Arrow points to electron-dense deposits (**c**, original magnification ×9300; **d**, original magnification ×4800). To optimize viewing of this image, please see the online version of this article at www.kidney-international.org.

In the Mayo Clinic cohort only 4 pediatric patients were tested, of which 1 case was positive for Sema3B. However, the combined French and Italian pediatric cohorts included a large number of patients and detected Sema3B in 6 of 59 pediatric MN patients (10.1%). Because 9 patients in each pediatric cohort had lupus class 5, the real prevalence of Sema3B-associated disease among nonlupus pediatric patients in France and Italy was 6 of 41 (14.6%). It is remarkable that 5 of 8 patients with Sema3B-positive MN were ≤2 years old; this makes Sema3B a new target antigen in young children with the disease. Interestingly, 2 patients had an autoimmune disease that included type 1 diabetes and thrombocytopenic purpura. One child at disease onset had a full-house immunofluorescence (patient 8 with no systemic or serologic features of lupus) that was not present in kidney biopsies performed at subsequent relapses.

The kidney biopsy findings of the glomeruli in Sema3B-associated MN were typical for MN. Granular Sema3B was present uniformly along the subepithelial region of the GBM, suggesting that Sema3B is shed from the podocytes rather than from the mesangial cells or endothelial cells because there is no mesangial or subendothelial staining for Sema3B. It is also likely that Sema3B does not represent trapped circulating antigen-antibody complexes because these are more likely to be present in the subendothelial region of the GBM and have a patchy irregular localization along the capillary walls, unlike the subepithelial uniform Sema3B staining in our cases. IgG subtypes were done in 4 cases and revealed IgG1 in 3 cases and IgG4 predominance along with IgG1 and IgG2 in 1 case. In addition, IgG4 (only) staining was done in all 4 cases of the Italian cohort, and all 4 cases were negative for IgG4, pointing to a non-IgG4 dominant IgG in Sema3B-associated MN, most likely IgG1. This is different from PLA2R-associated MN where IgG4 is the dominant IgG subclass.

Interestingly, tubular basement membrane deposits were present in 4 pediatric cases. All 4 cases had onset < 2 years and showed tubular basement membrane deposits that stained for IgG. Of note, the tubular basement membrane deposits were negative for Sema3B staining, although glomerular deposits were brightly stained for Sema3B in the same sections (Figure 7, Supplementary Figure S3). Lupus serologies were evaluated and were negative in all cases. Thus, tubular basement membrane deposits in the setting of MN in a child < 2 years old may indicate a Sema3B-associated MN. The finding of Sema3B-associated MN in the pediatric age group, in particular in very young patients, and the fact that in the Italian cohort patients 7 and 8 are siblings and patient 6 has a father with MN raise the possibility of a genetic basis of the disease. Further studies are needed to determine whether alterations in the sequence of the protein enhancing its antigenicity or specific immune response genes (human leukocyte antigen D allotypes) are involved.

Semaphorins are a group of secreted and transmembrane/membrane bound proteins containing a conserved extracellular Semaphorin (sema) domain of about 500 amino acids characterized by highly conserved cysteine residues.[8–10] The sema domain is the critical component through which semaphorins mediate their effects. The first semaphorins were identified as proteins that guided neuronal axons to their targets. Since then more than 20 semaphorins have been identified and are classified into 8 subclasses: Classes I, and IV to VII are membrane-associated (I, IV, V, and VI are transmembrane and VII is membrane bound), whereas classes II, III, and VIII are secreted. In addition to the nervous system, semaphorins are also widely expressed in other organ systems

including cardiovascular, endocrine, gastrointestinal, hepatic, immune musculoskeletal, renal, reproductive, and respiratory systems. The primary semaphorin receptors are the receptors plexins and neuropilins; plexins consist of 4 subclasses A through D and neuropilins consist of neuropilin 1 and 2. The expression of various semaphorins is diverse in different organ systems, suggesting they serve varying functions during development and in adults. Semaphorins also have a role in immune system by modulating cytoskeletal organization.[11] Furthermore, the semaphorins also play a role in disease pathogenesis including tumorigenesis and metastasis, retinal degeneration, rheumatoid arthritis, and decreased bone mineral density.[12] However, the exact mechanisms, function, and disease association (if any) of most semaphorins remains unknown.

Semaphorin 3 is a secreted protein with a sema domain, PSI (plexin-semaphorin-integrin) domain, Ig domain, and a basic domain (Figure 6b). Semaphorin 3 and receptors have been detected in endothelial cells, podocytes, and tubular epithelial cells.[8,13] In particular Sema3A has been shown to regulate slit diaphragm proteins such as podocin and decrease its interaction with CD2-associated protein and nephrin. Sema3A also induced podocyte apoptosis.[8] The role and function of Sema3B in the kidney is not known, and to the best of our knowledge overexpression of Sema3B has not been reported in any kidney disease.

Unexpectedly our results show reactivity of Sema3B antibodies only under reducing conditions. This could suggest that the epitope is unmasked by disruption of the disulfide bonds. Sema3B contains conserved cysteine residues forming intrasubunit disulfide bonds. In some conditions early in life, disruption of these disulfide bonds exposes new protein domains that could serve as neoepitopes. It would be interesting to determine the 3-dimensional structure of Sema3B to localize the site of conformational change and to determine whether reduced conditions are required for the antibodies to recognize Sema3B throughout the course of the disease. These *de novo* created structures could lead to autoimmunization. It is tempting to speculate that the anti-Sema3B antibodies are formed to an epitope on the sema domain or even the basic domain because MS detected extensive sequence coverage of these segments of the Sema3B protein (Figure 2B).

Because this is a retrospective study, limitations include sera availability in only 5 patients and complete IgG subtypes performed in only 4 patients. There are also important questions that future studies need to address: Why tubular basement membrane staining for IgG is present in the very young patients? Why is the tubular basement membrane staining negative for Sema3B? Is the antigen in the tubular basement membrane deposits different from Sema3B present in the MN deposits? Is the Sema3B-associated MN different in pediatric patients compared with adult patients?

Circulating antibodies against the recombinant protein were detected by Western blot in patients with active disease, and they disappear on clinical remission. These antibodies were present in Sema3-associated MN patients who were negative for anti-PLA2R, anti-THSD7A, and anti–NELL-1 antibodies and thus not reflective of a secondary immunologic phenomenon.[14] Further studies are required to confirm whether Sema3B is a true antigen or merely a biomarker in Sema3B-associated MN. These include studies that localize the Sema3B on podocyte surface and studies that determine the specificity of circulating antibodies to glomerular Sema3B.

In conclusion, Sema3B-associated MN represent a unique type of primary MN that is more likely to be present in pediatric patients, particularly in very young patients with onset <2 years old. Sema3B-associated MN should be added to the list of serologically defined MN, including PLA2R-, THSD7A-, and NELL-1–associated MN.

METHODS

Patient selection

Mayo Clinic cohort. We performed MS/MS in selected kidney biopsies of 70 cases of PLA2R-negative MN received at the Mayo Clinic (2015–2018) for analysis by MS/MS and detected the unique protein, Sema3B, in 2 cases. The 70 cases included both primary and secondary membranous nephritis. These cases were also used to detect recently reported novel antigens EXT1/EXT2 and NELL1.[6,7] IHC was then performed to confirm the MS/MS results. In addition, we screened 160 PLA2R-negative MN cases (including the 68 negative and 2 positive on MS/MS) by IHC for Sema3B. We detected only 1 more positive case, which was confirmed by MS/MS. Other than 4 pediatric cases (<18 years of age), the remaining cases were all adults.

French and Italian validation cohorts. One hundred eighteen biopsies were stained for Sema3B, of which 59 were from the French (adult) cohort 1, 43 from the Italian pediatric cohort, and 16 from the French (pediatric) cohort 2. All cases were PLA2R-, THSD7A-, EXT1/EXT2-, and NELL-1–negative MN. Each pediatric cohort included 9 lupus class V cases.

Control cases. For control cases, we performed MS/MS on 111 cases that included 15 cases of time 0 transplant biopsies, 17 cases of minimal change disease, 44 cases of focal segmental glomerulosclerosis, 7 cases of diabetic glomerulosclerosis, 5 cases of IgA nephropathy, and 23 cases of PLA2R-positive MN. For control IHC we used 45 cases: 9 cases of minimal change disease, 9 cases of focal segmental glomerulosclerosis, 4 cases of IgA nephropathy, 7 cases of diabetes, 15 cases of PLA2R-positive MN, and 1 case of time 0 transplant protocol biopsy.

These biopsies were received in the Renal Pathology Laboratory, Department of Laboratory Medicine and Pathology, Mayo Clinic for diagnosis and interpretation between January 2015 and May 2018. Light microscopy, IF including PLA2R studies, and electron microscopy were performed in each case of MN. The clinical information was obtained from the accompanying charts. The study was approved by the Mayo Clinic Institutional Review Board.

Laser microdissection and MS: protein identification by laser capture microdissection, trypsin digestion, and nano-liquid chromatography orbitrap MS/MS

For each case 10-μm-thick formalin-fixed paraffin sections (FFPE) were obtained and mounted on a special polyethylene naphthalate membrane laser microdissection slide and using a Zeiss Palm Microbean microscope (White Plains, NY); the glomeruli were microdissected to reach approximately 250,000 to 500,000 μM^2 per

case. Resulting FFPE fragments were digested with trypsin and collected for MS/MS analysis. The trypsin digested peptides were identified by nano-flow liquid chromatography electrospray MS/MS using a Q-Exactive mass spectrometer (Thermo Fisher Scientific, Bremen, Germany) coupled to an Ultimate 3000 RSLCnano HPLC system (Thermo Fisher Scientific). All MS/MS samples were analyzed using Mascot and X! Tandem set up to search a Swissprot human database. Scaffold (version 4.8.3, Proteome Software Inc., Portland, OR) was used to validate MS/MS-based peptide and protein identifications. Peptide identifications were accepted at greater than 95.0% probability by the Scaffold Local FDR algorithm with protein identifications requiring a 2-peptide minimum and a 95% probability using Protein Prophet.[15]

Immunohistochemical staining for Sema3B

Tissue sectioning and IHC staining was performed at the Pathology Research Core (Mayo Clinic, Rochester, MN) using the Leica Bond RX stainer (Leica). FFPE tissues were sectioned at 5 μm, and IHC staining was performed on-line. Slides for Sema3B stain were retrieved for 20 minutes using Epitope Retrieval 1 (Citrate; Leica) and incubated in Protein Block (Dako) for 5 minutes. The Sema3B primary antibody (Rb. Polyclonal; Abcam #ab48197) was diluted to 1:200 in Background Reducing Diluent (Dako) and incubated for 15 minutes. The detection system used was Polymer Refine Detection System (Leica). This system includes the hydrogen peroxidase block, post primary and polymer reagent, DAB, and hematoxylin. Immunostaining visualization was achieved by incubating slides 10 minutes in DAB and DAB buffer (1:19 mixture) from the Bond Polymer Refine Detection System. To this point, slides were rinsed between steps with 1× Bond Wash Buffer (Leica). Slides were counterstained for 5 minutes using Schmidt hematoxylin and molecular biology grade water (1:1 mixture), followed by several rinses in 1× Bond wash buffer and distilled water (this is not the hematoxylin provided with the Refine kit). Once the immunochemistry process was completed, slides were removed from the stainer and rinsed in tap water for 5 minutes. Slides were dehydrated in increasing concentrations of ethyl alcohol and cleared in 3 changes of xylene before permanent cover slipping in xylene-based medium.

Immunofluorescence staining for Sema3B and co-localization analysis

Immunofluorescence staining was performed on FFPE sections retrieved for 30 minutes using target retrieval solution high pH (Dako) in pressure cooker equipment (BioSB). The Sema3B primary antibody (rabbit polyclonal, Abcam antibodies) was diluted to 1:100 in blocking solution (2% calf fetal serum and 2% normal goat serum) and incubated overnight at 4°C with retrieved biopsy sections. Next, the slides were incubated with secondary antibody the goat Alexa488-conjugated anti-rabbit Fab IgG antibodies (dilution 1:400; Life Technologies). Next, anti-human IgG Alexa Fluor 647 rabbit monoclonal antibody (dilution 1:400; Sigma) was reacted with the retrieved tissue after staining for Sema3B as described above. Finally, slides were mounted in mounted medium (Thermo Scientific) and covered with LDS2460EP cover slips. Co-localization of Sema3B and IgG along the GBMs was examined by confocal microscopy using a Leica TCS-SP2 and analyzed with Leica Confocal Software version 2.61 (Wetzlar, Germany).

Western blot analysis

Sema3B recombinant human protein (AVIVA System Biology) was diluted with nonreducing or reducing Laemmli sample buffer (Bio-Rad) and boiled for 10 minutes. Samples were loaded into Criterion 4% to 15% TGX gels (Bio-Rad) and electrophoresed in Tris-glycine-SDS running buffer. Proteins were transferred to polyvinylidene difluoride membranes according to standard protocols, and then membranes were blocked with Pierce Protein-Free Blocking buffer (Thermo Scientific). Membranes were incubated overnight at 4 °C with sera from patients, control subjects (dilution 1:50), and rabbit polyclonal antibodies (dilution 1:2000) against Sema3B (Abcam). Subsequently, blots were washed and incubated for 2 hours at room temperature with goat anti-human or goat anti-rabbit IgG, AP conjugate (both dilution 1:10,000; Sigma). Immunoreactive proteins were visualized with BCIP/NBT liquid substrate system (Sigma).

DISCLOSURE

All the authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

SS and FCF designed the study. SS wrote the manuscript and interpreted the kidney biopsy, clinical, IHC, and MS data. BM and CC performed the laser microdissection and mass spectrometry. AR helped in gathering clinical data. LG performed the IHC. HD, DB, and FDC provided tissue for the validation cohorts. HD performed the immunofluorescence, confocal studies, and Western blot analysis. PR, MV, FE, and TU provided clinical information. The manuscript was drafted and written by the first author, with input as appropriate from the investigators.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Supplementary Methods. Details of laser microdissection and mass spectrometry.

Figure S1. An example of MS/MS spectra match to a sequence from Sema3B.
Figure S2. Confocal microscopy of additional Semaphorin 3B–positive MN case.
Figure S3. Immunofluorescence staining for IgG and Semaphorin 3B in additional cases.
Table S1. Immunofluorescence staining in Semaphorin 3B positive cases.

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