Recurrence of Anti-Semaphorin 3B–Mediated Membranous Nephropathy after Kidney Transplantation

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ABSTRACT

Background Membranous nephropathy (MN) is rare in pediatric patients, although its diagnosis may be underestimated in children who are responsive to corticosteroid therapy prescribed for a suspicion of minimal change disease. It is most often associated with an autoimmune disease, predominantly lupus. We previously reported the occurrence of early-onset MN associated with semaphorin 3B in nine children and two adults.

Methods Biopsies were performed on native kidney and at 1 and 5 months after transplantation. Semaphorin 3B antigen was detected in immune deposits by immunohistochemistry and confocal microscopy on paraffin-embedded biopsies. Anti-semaphorin antibodies were detected by Western blot and analyzed sequentially.

Results We report the first case of early recurrence after transplantation in a 7-year-old boy who presented with severe nephrotic syndrome and advanced kidney failure. There was no evidence of hereditary or associated autoimmune disease. Abundant, almost coalescent deposits were seen by electron microscopy and bright granular, subepithelial staining was observed for semaphorin 3B antigen. Western blot analysis of serum revealed anti-semaphorin 3B antibodies. Recurrence of MN occurred 25 days after transplantation and manifested as nephrotic range proteinuria despite conventional immunosuppressive therapy. Kidney biopsies confirmed histologic MN recurrence with colocalization of semaphorin 3B antigen and IgG. The patient was treated with rituximab. Anti-semaphorin 3B antibodies, which were detected at transplantation, were not detected 40 days after rituximab.

Conclusion This case provides evidence that anti-semaphorin 3B antibodies are pathogenic and should be monitored in patients with MN.

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Membranous nephropathy (MN) in an uncommon cause of nephrotic syndrome in pediatric patients as compared with adults, being most frequently associated with an autoimmune disease such as lupus, more rarely with an infection (hepatitis B) or a malignancy. It is remarkable that the first podocyte antigen identified in humans in 2002 was neutral endopeptidase in a rare subset of children with antenatal MN.[1] This discovery was followed by the identification in 2009 of the main target antigen in the adult, the M-type phospholipase A2 receptor (PLA2R),[2] followed by that of thrombospondin type I domain containing 7A (THSD7A).[3] In patients with PLA2R-associated MN, positive serology now tends to replace kidney biopsy and quantitative assessment of PLA2R antibody trajectory is helpful in predicting response to therapy.4 Thanks to the technological leap combining laser microdissection of glomeruli and mass spectrometry identification of solubilized digested proteins, several additional candidate antigens have been identified in the last 2 years, such as protein kinase C-binding protein NELL1 (also known as neural epidermal growth factor like 1 protein), semaphorin 3B (SEMA3B), neural cell adhesion molecule 1 (NCAM1), protocadherin 7 (PCDH7), and serine protease HTRA1.[5,6] Among these "new" antigens, semaphorin 3B is characteristic of pediatric patients. Eight of the 11 patients with semaphorin 3B–associated MN

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were pediatric patients, and in five patients the disease started at or below the age of 2 years.[7] As for the other new candidate antigens, the question remains whether semaphorin 3B is a *bona fide* antigen or a biomarker, and whether anti-semaphorin 3B antibodies are pathogenic. This question is not only very important for the management of these rare patients but also for understanding the pathophysiology of those diseases. Here, we report the first case of a pediatric patient with a very early recurrence of semaphorin 3B–related MN after kidney transplantation, strongly supporting the pathogenicity of anti-semaphorin 3B antibodies. Treatment with rituximab led to a complete clinical remission within 5 months after MN recurrence. Monitoring semaphorin 3B antibody in the serum seems to be a promising, noninvasive tool to guide diagnosis of disease recurrence and management of the patient.

METHODS

Detection of Semaphorin 3B Antigen and IgG in Paraffin-Embedded Kidney Biopsy Specimens and Colocalization Analysis

Immunofluorescence staining was performed on formalin-fixed paraffin-embedded sections retrieved for 30 minutes using target retrieval solution high pH (Dako) in pressure cooker equipment (Bio SB). The semaphorin 3B primary antibody (rabbit polyclonal, Abcam antibodies) was diluted to 1:100 in blocking solution (2% FCS and 2% normal goat serum) and incubated overnight at 4°C with retrieved biopsy sections. Next, the slides were incubated with a secondary antibody: Alexa488-conjugated goat anti-rabbit Fab IgG antibody (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 semaphorin 3B as described above. Finally, slides were mounted in mounting medium (Thermo Scientific) and covered with LDS2460EP coverslips. Colocalization of semaphorin 3B and IgG along the glomerular basement membrane was examined by confocal microscopy using a Leica TCS-SP2 and analyzed with Leica Confocal Software version 2.61 (Wetzlar, Germany).

Western Blot Analysis

Semaphorin 3B recombinant human protein (Aviva Systems Biology) was diluted with reducing Laemmli sample buffer (Bio-Rad) and boiled for 10 minutes. Samples were loaded into Criterion 4%–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 semaphorin 3B (Abcam). Subsequently, blots were washed and incubated for 2 hours at room temperature with goat anti-human or goat anti-rabbit IgG, alkaline phosphatase conjugate (both dilutions 1:10,000; Sigma). Immunoreactive proteins were visualized with BCIP/NBT liquid substrate system (Sigma).

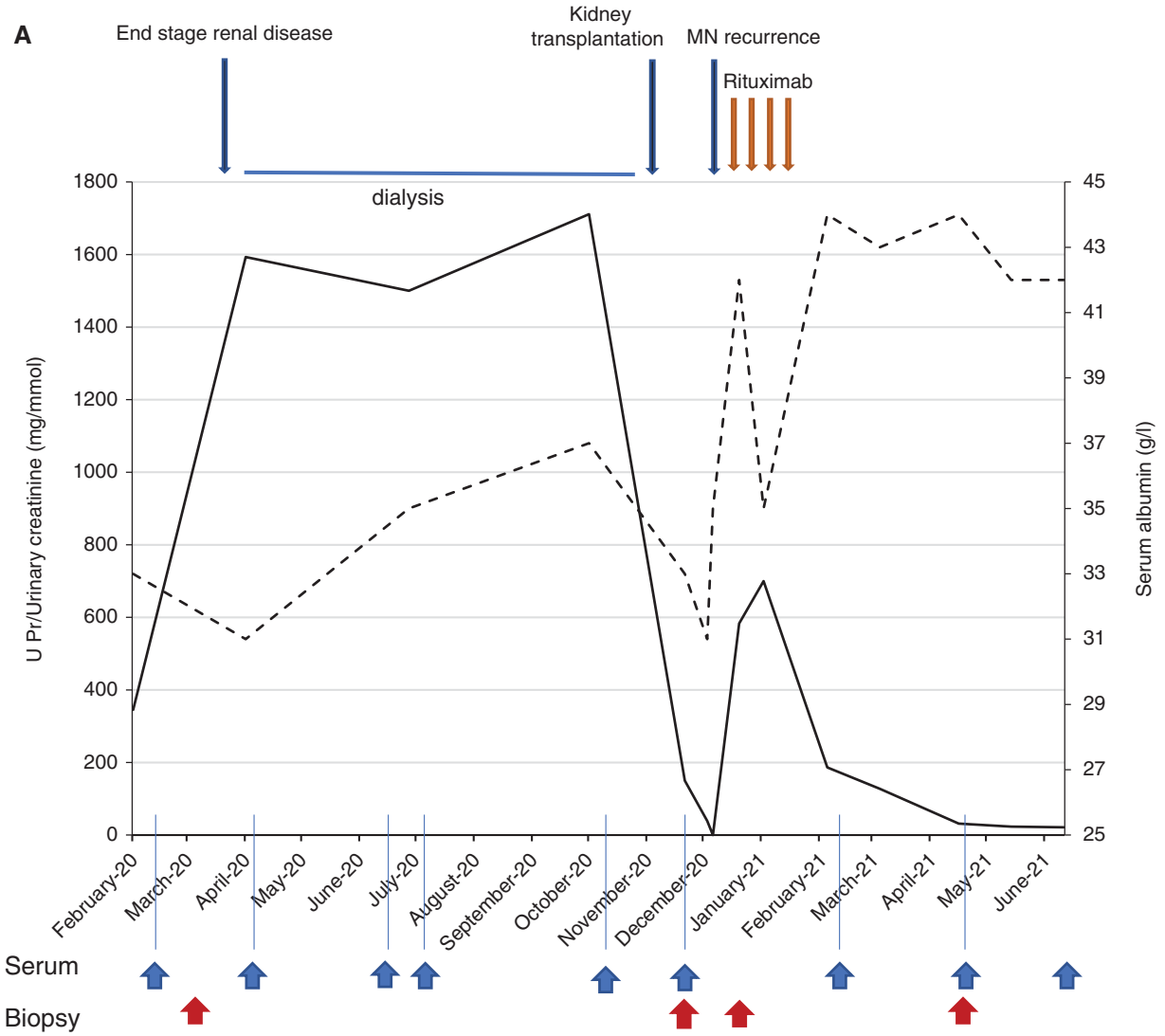
RESULTS

A 7-year-old boy was referred to our center in Montpellier, France by Georgian pediatricians for a refractory primary MN. No family history of kidney disease was reported. Nephrotic syndrome occurred in 2014. No treatment was then started. A kidney biopsy performed in 2015 showed MN stage III with granular IgG deposits along the glomerular capillary walls. Immunostaining for PLA2R and THSD7A was negative. Anti-PLA2R antibodies were not detected. First-line therapy included corticosteroids, cyclophosphamide, and angiotensin-converting enzyme inhibitors and was ineffective on proteinuria. Two years later, tacrolimus was started without any efficacy on the proteinuria level.

> **Significance Statement**
>
> We report the first case of early recurrence after transplantation of membranous nephropathy associated with antibodies directed at semaphorin 3B, a recently described putative antigen. This case provides strong evidence that the disease is caused by anti-semaphorin 3B antibodies entering the graft from the recipient circulation. It also suggests that these antibodies are a new biomarker of the disease that should be carefully monitored before and after transplantation. Finally, the finding supports the efficacy of rituximab.

On admission in February 2020, the patient had CKD stage 4 (serum creatinine 157 μmol/L, eGFR according to Schwartz formula was 26 ml/min per 1.73m^2), with serum albumin 3.3 g/dl and nephrotic range proteinuria (2.45 g/L, urine protein/creatinine ratio 345 mg/mmol) (Figure 1). All causes of secondary MN including autoimmune disease, infections, drugs, and malignancy were excluded. Search for circulating anti-PLA2R and anti-THSD7A antibodies was negative. A kidney biopsy was performed, revealing severe glomerular sclerosis (>50% glomeruli) associated with extensive interstitial fibrosis and tubular atrophy (>30%).

Due to the coronavirus disease pandemic and given the severity of kidney lesions, rituximab was not started and only conservative care was maintained. ESKD occurred within 2 months, complicated with a cardiac tamponade and a pleural effusion that required pericardiocentesis and an urgent dialysis start. On dialysis, the patient had unusually persistent diuresis with nephrotic range proteinuria, which led to a unilateral nephrectomy being performed on the day of kidney transplantation. Strong subepithelial staining for IgG was detected along glomerular capillary walls, associated with granular staining along the tubular basement membranes. Electron microscopy showed very abundant, almost contiguous subepithelial electron dense deposits (Figure 2). The



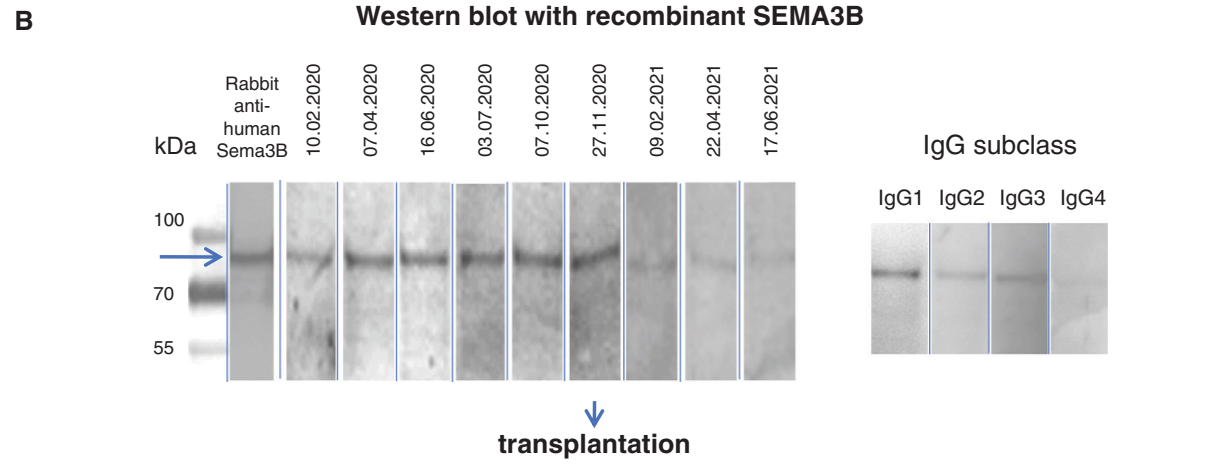


Figure 1. Time course of clinical and immunologic activity. (A) Time course of clinical parameters, treatment and sample collection. (B) Detection of anti-Semaphorin 3B protein antibodies in the serum by Western blot. All incubations of individual strips were performed at the same time in the same experiment at the end of follow-up when all samples were available. Note decrease of antibodies after transplantation and RTX treatment.

nephrectomy specimen was then stained for potential antigens. Although immunostaining for PLA2R, NELL1, EXT1, and EXT2 was negative, a strong positive, granular staining for semaphorin 3B was revealed along the glomerular capillary walls. By confocal microscopy, semaphorin 3B and IgG staining showed impressive colocalization in immune deposits (Figure 2).

Kidney transplantation from a deceased pediatric donor was successfully

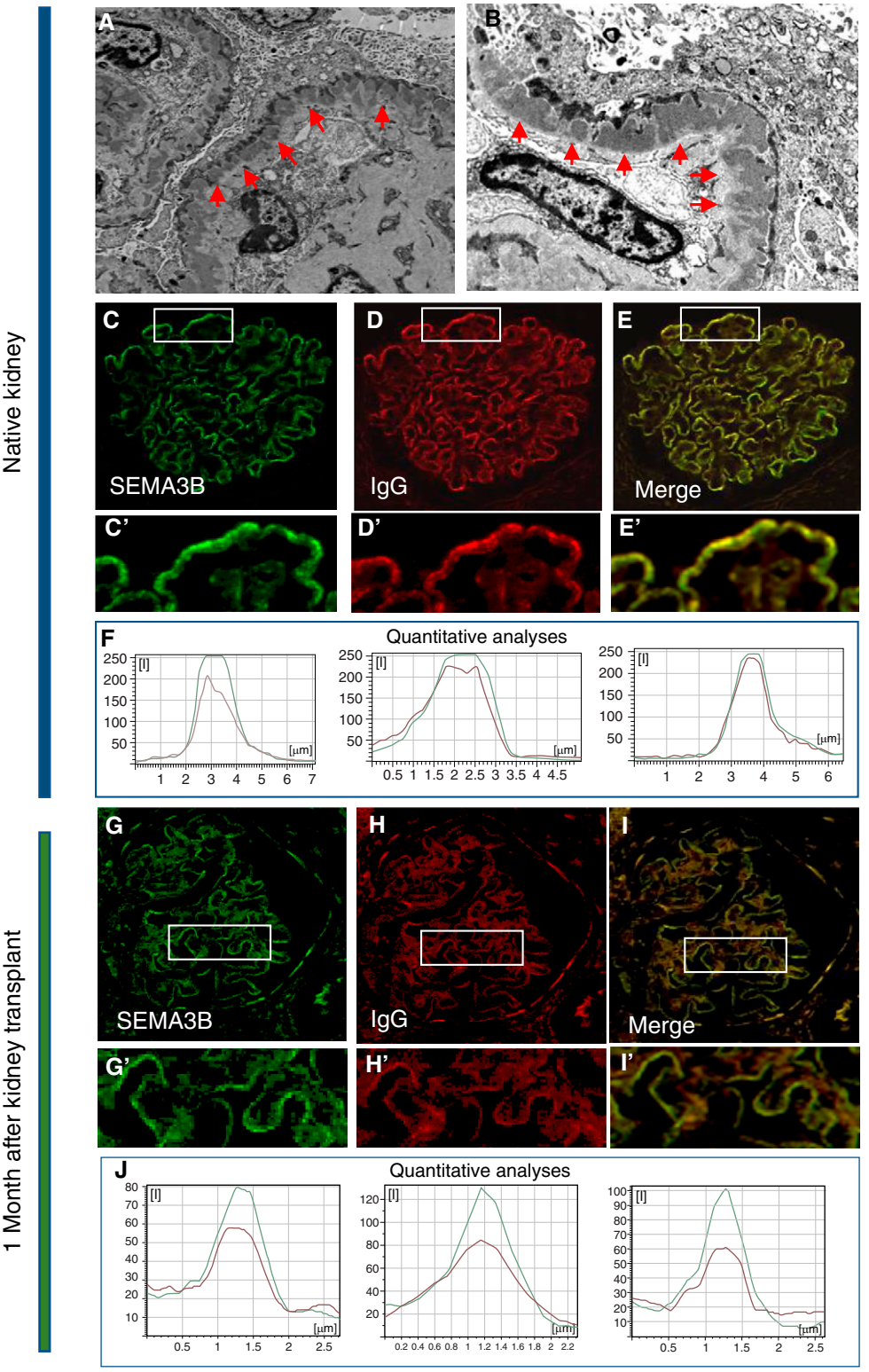


Figure 2.

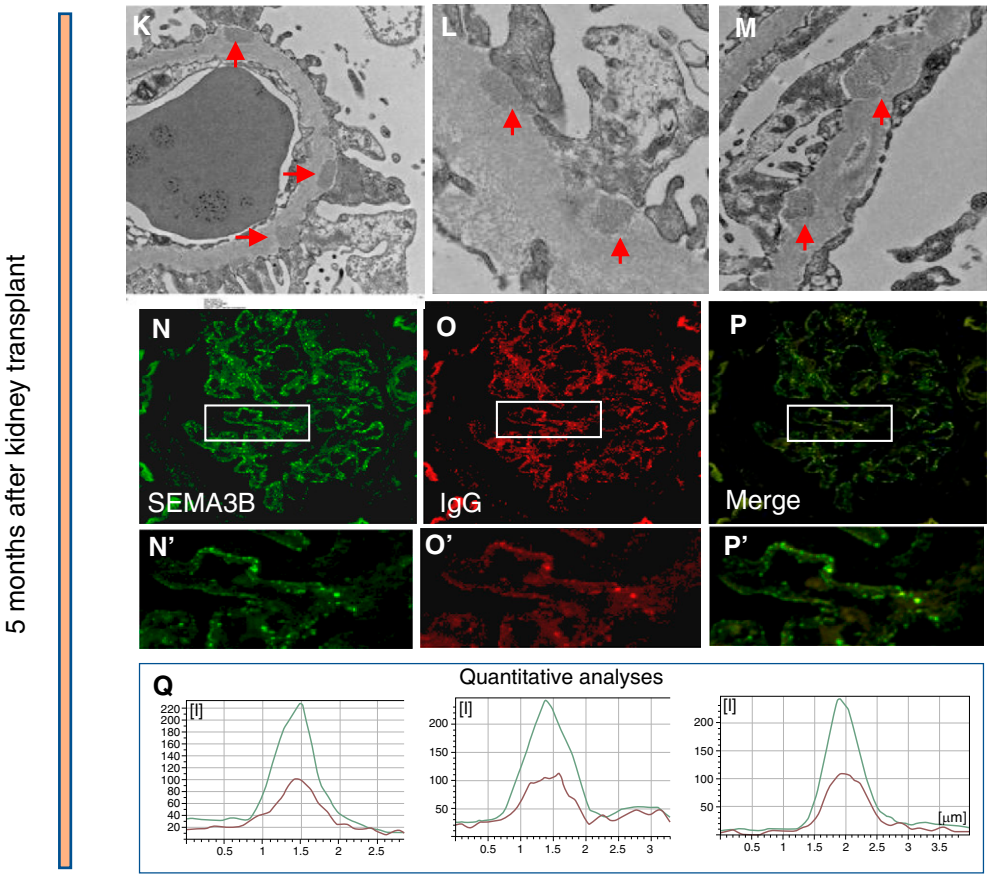


Figure 2. Semaphorin 3B (SEMA3B) protein localized to immune deposits in glomerular membrane. (A–F) Nephrectomy specimen of native kidney. (A and B) Representative segment of the capillary wall containing immune deposits revealed as electron dense material localized in the basement membrane by electron microscopy (red arrows). (C–E) Detection of SEMA3B protein and IgG in glomerular immune deposits by confocal immunofluorescence microscopy analysis. Biopsy was double-labeled with (C) anti-SEMA3B (green) and (D) anti-human IgG (red). (E) Merged image of (C) and (D). (C′–E′) Enlarged images of the boxed areas in (C), (D), and (E) respectively. (F) Graphs showing quantitative analysis of the fluorescence recorded across sections of a representative capillary loop. Note the superimposition of the two signals, which indicates that subepithelial immune deposits are composed of SEMA3B (green) and IgG (red). (G–J) Kidney graft biopsy performed 1 month after transplantation. (G–I) Confocal microscopy showed weak, granular deposits of SEMA3B which colocalized with IgG. Biopsy was double-labeled with (G) anti-SEMA3B (green) and (H) anti-human IgG (red). (I) Merged image of (G) and (H). (G′–I′) Enlarged images of the boxed areas in (G), (H), and (I) respectively. (J) Graphs showing quantitative analysis of the fluorescence recorded across sections of a representative capillary loop. (K–Q) Kidney graft biopsy performed 5 months after transplantation. (K–M) Electron microscopy showed small subepithelial electron dense deposits, mostly located by the podocyte sole with areas of foot process effacement (red arrows). (N–P) Confocal microscopy showed persistent weak subepithelial deposits of SEMA3B which colocalized with IgG staining. (N′–P′) Enlarged images of the boxed areas in (N), (O), and (P) respectively. (Q) Graphs showing quantitative analysis of the fluorescence recorded across sections of a representative capillary loop.

performed in November 2020. Immunosuppressive regimen included an induction with basiliximab 10 mg at day 1 and day 4 in association with a pulse of corticosteroids (300 mg/m^2), Maintenance immunosuppressive regimen included corticosteroids (60 mg/m^2 per day), tacrolimus (0.3 mg/kg per day), and mycophenolate mofetil (1200 mg/m^2 per day). On day 14, serum creatinine, serum albumin, and proteinuria/urinary creatinine ratio levels were 56 µmol/L, 3.5 g/dl, and <20 mg/mmol, respectively.

On day 25, isolated nephrotic range proteinuria (2.45 g/L, urine protein/creatinine ratio 583 mg/mmol) appeared. A kidney transplant biopsy was then performed. Light microscopy examination was normal but immunofluorescence analysis revealed finely granular, subepithelial IgG deposits with rare C3 and no C1q deposits, posing the diagnosis of MN recurrence. Electron microscopy was not performed. Confocal microscopy showed weak, granular deposits of semaphorin 3B which colocalized with IgG (Figure 2).

Treatment with rituximab was then started (four infusions, 375 mg/m^2 each week) in association with renin-angiotensin-aldosterone system blockers (enalapril). B cell depletion was obtained after the first infusion. Proteinuria dramatically decreased within 2 months and complete remission was obtained within

4 months (Figure 1). The immunosuppressive regimen was not changed: corticosteroids (tapering to a daily dose of 7.5 mg), mycophenolate mofetil (target area under the concentration time curve concentration of mycophenolate acid 30–60 mg· h/L), and tacrolimus (target through level of 6–8 ng/ml). A protocol kidney transplant biopsy was performed at 5 months after kidney transplant, revealing persistent subepithelial deposits of IgG1 which colocalized with semaphorin 3B staining. Other IgG subclasses, C3 and C1q, were absent. Electron microscopy showed small subepithelial electron dense deposits, mostly located by the podocyte sole with areas of foot process effacement (Figure 2).

At 6 months, eGFR according to Schwartz formula was 72 ml/min per 1.73m^2. The patient has sustained complete, clinical remission (urine protein/creatinine ratio 22 mg/mmol, urinary albumin/creatinine ratio 0.6 mg/mmol) with a normal serum albumin level (4.2 g/dl). Complete B cell depletion has been maintained to date.

We retrospectively asked whether anti-semaphorin 3B antibody reactivity in serum paralleled clinical activity, and thus could serve as biomarker for the monitoring of the patient. We retrieved all available sera at various time points, which we incubated with reduced recombinant semaphorin 3B. We found a strong signal until transplantation (November 27, 2020) which rapidly dropped thereafter (Figure 1).

At 1 year after transplantation (11 months after recurrence of MN), the patient had a sustained complete immunologic (absence of anti-semaphorin 3B antibodies) and clinical (urine protein/creatinine ratio 21 mg/mmol, urinary albumin/creatinine ratio 6.1 mg/mmol, and serum albumin level 4.4 g/dl) remission.

DISCUSSION

We report the first case of early recurrence of semaphorin 3B–associated MN on the graft. The patient presented all features of semaphorin 3B–associated MN, including onset at a very early age (1 year), predominance of IgG1 subclass in deposits, granular deposits along the tubular basement membrane, colocalization of semaphorin 3B and IgG in subepithelial immune deposits, and presence of anti-semaphorin 3B antibodies in serum.

This case is important both from a pathophysiologic and clinical point of view. Six putative antigens have been discovered in the past 2 years using a combination of laser microdissection of glomeruli and mass spectrometry of digested proteins: EXT1 and EXT2 in 2019[8]; NELL1[9] and semaphorin 3B[7] in 2020; and PCDH7,[10] HTRA1,[11] and NCAM1[12] in 2021. EXT1 and EXT2 are found in the deposits in one third of patients with lupus MN but no antibody was detected as yet despite diligent efforts.[8] Whether these new candidate antigens are *bona fide* antigens or biomarkers is controversial. Their identification from the biopsy specimen has led to question the minimal definition of an antigen which requests the presence of the relevant antibodies in the blood and ideally in biopsy samples, the usual absence of antibodies in other immunopathologic settings such as PLA2R-associated MN, and the parallel outcome of antibody levels with clinical activity. Although colocalization of antigen and antibody by confocal microscopy is suggestive, definitive evidence of the reactivity of the deposited antibody against the potential antigen requires elution experiments which could not be achieved in our initial study because of the lack or the small size of frozen tissue specimen particularly in children.[7] Here, early recurrence of the disease within 1 month as has been observed in PLA2R-[13,14] and THSD7A-associated MN,[15] is a strong argument favoring a pathogenic role of anti-semaphorin 3B. Moreover, the biopsy of the native kidney and the two biopsies of the graft showed similar colocalization of semaphorin 3B and IgG[1] in the immune deposits. The rapid drop in the anti-semaphorin 3B level after transplantation suggests a combination of a sink effect as the graft offers new semaphorin 3B antigen to the circulating antibody and of the therapeutic effect of rituximab. Transplantation represents a unique opportunity to understand the immunologic initiation phase provided that kidney biopsy is performed early. During this phase, the antibody starts to accumulate in immune deposits, yielding a weak but clear granular, subepithelial fluorescence as we observed 1 month after transplantation. Unfortunately, we could not perform electron microscopy at this early phase. Overall, these findings strongly support a pathogenic role for anti-semaphorin 3B antibodies, which should be confirmed by the development of an experimental model. More than a decade after the discovery of PLA2R, the only experimental model of PLA2R-associated MN is with the mouse antigen,[16] and pathogenicity mostly relies on early recurrence after transplantation[13,14] and decrease of PLA2R antibody levels preceding proteinuria, all findings now observed in semaphorin 3B–associated MN, albeit in a low number of patients.

From a clinical point of view, this report is a strong incentive to assess anti-semaphorin 3B antibodies in children who are candidates for transplantation. For the time being, this can only be done by Western blot in expert centers. Hopefully ELISA or immunofluorescence assays will be developed for detection of antibodies specific for semaphorin 3B and other new putative antigens. The finding of anti-semaphorin 3B antibodies has therapeutic implications; when present at transplantation, these antibodies may predict recurrence on the graft. Of note, nephrotic syndrome developed despite immunosuppressive therapy to control graft rejection, whereas addition of rituximab was associated with complete remission of the nephrotic syndrome. These findings suggest a therapeutic benefit of an early switch to rituximab as observed in PLA2R-associated MN.[4]

In summary, we have reported the first case of recurrence of semaphorin 3B–associated MN, suggesting that the specific antibodies are pathogenic. This case report also suggests that rituximab is an efficient therapy in this setting

and that anti-semaphorin 3B antibodies should be monitored in these patients.

DISCLOSURES

M. Fila reports honoraria with Alexion and Genzyme. P. Ronco reports consultancy with Alexion and MorphoSys; reports honoraria with Alexion, MorphoSys, Northwell, Podocyte meeting, and Vanderbilt; and reports advisory or leadership role with Alexion, Amicus, and MorphoSys. All remaining authors have nothing to disclose.

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AUTHOR CONTRIBUTIONS

M. Fila, H. Debiec, and P. Ronco designed the study; H. Debiec, H. Perrochia, and M.-C. Verpont carried out the experiments; M. Fila, H. Debiec, H. Perrochia, M.-C. Verpont, D. Buob, and P. Ronco analyzed the data; M. Fila and H. Debiec made the figures; M. Fila, H. Debiec, H. Perrochia, D. Buob, and P. Ronco drafted and revised the paper; and all authors approved the final version of the manuscript.

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