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C5b-9 Glomerular Deposits Are Associated With Poor Renal Survival in Membranous Nephropathy



Maxime Teisseyre^{1,2}, Anaïs Beyze^{1,2}, H  l  ne Perrochia³, Ilan Szwarc¹, Alexis Bourgeois¹, Coralie Champion¹, Leila Chenine¹, Jean-Emmanuel Serre¹, Jonathan Broner⁴, C  dric Aglae¹, Vincent Pernin^{1,2} and Moglie Le Quintrec^{1,2}

¹Department of Nephrology, Dialysis and Transplantation, Lapeyronie Hospital, Montpellier University Hospital, University of Montpellier, Montpellier, France; ²Institute for Regenerative Medicine and Biotherapy, Institut National de la Sant   Et de la Recherche M  dicale, Montpellier University Hospital, University of Montpellier, Montpellier, France; ³Department of Pathology, Gui de Chauliac Hospital, Montpellier University Hospital, University of Montpellier, Montpellier, France; and ⁴Department of Internal Medicine, Caremeau Hospital, N  mes University Hospital, University of Montpellier, N  mes, France

Introduction: Membranous nephropathy (MN) is the first cause of nephrotic syndrome in patients without diabetes. Its prognosis is variable, and treatment remains controversial because of potential toxicity. Currently, there is no reliable prognostic marker common to all etiologies of MN and routinely available to predict the disease course and guide therapeutic management. Despite the major role of complement in the glomerular damage of MN, its prognostic impact has never been studied. We investigated the frequency and prognostic impact of glomerular deposition of C5b-9 in MN.

Methods: We retrospectively selected adults diagnosed with MN (primary or secondary) at Montpellier University Hospital between December 2004 and December 2015. To be included, all patients were required to have complete medical data and a kidney tissue sample for further immunohistochemistry. We performed *PLA2R1*, C4d, and C5b-9 staining by immunohistochemistry.

Results: Sixty-four adults were included: 45 with primary MN and 19 with secondary MN. C4d was positive in the glomeruli of 61 adults (95.3%). Twenty-nine adults (45.3%) had glomerular deposition of C5b-9. Patients with glomerular deposition of C5b-9 had more severe nephrotic syndrome on diagnosis and lower remission and renal survival rates than adults without.

Conclusion: C5b-9 glomerular staining is a powerful and easily accessible tool for stratifying adults according to their renal prognosis. The efficacy of complement inhibitors should be tested in adults with glomerular deposition of C5b-9.

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KEYWORDS: chronic renal failure; complement; membrane attack complex; membranous nephropathy; nephrotic syndrome; proteinuria

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Membranous nephropathy (MN) is a glomerular disease and a leading cause of nephrotic syndrome. MN can be primary (pMN), i.e., occurring in the absence of an established cause (70%–80% of cases), or secondary (sMN) to clinical disorders such as

Correspondence: Moglie Le Quintrec, Department of Nephrology, Dialysis and Transplantation, University of Montpellier, Institute of Regenerative Medicine and Biotherapy, Institut National de la Sant   Et de la Recherche M  dicale U1183, Centre Hospitalier Universitaire de Montpellier–H  pital Lapeyronie, 371, Avenue du Doyen Gaston Giraud, 34295 Montpellier Cedex 5, France. E-mail: m-lequintrec-donnette@chu-montpellier.fr

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infectious diseases, autoimmune disorders, cancer, and drug side effects. On light microscopy, MN is characterized by a pathologic change of the glomerular basement membrane caused by the accumulation of immune complexes, which appear as granular deposits of immunoglobulin (Ig) G and complement proteins on immunofluorescence.¹ The involvement of complement in this pathology has long been demonstrated.^{2–5} The formation of subepithelial immune deposits and the resulting complement activation is responsible for the functional impairment of the podocyte, causing nephrotic syndrome. The course of the disease is highly variable, ranging from spontaneous remission to end-stage renal disease.⁶ Consequently, treatment with costly and potentially toxic drugs remains controversial

and challenging. The key to more personalized care is the identification of reliable biomarkers. Interestingly, in antibody-mediated rejection of kidney allograft, another complement-mediated disease, glomerular deposition of C5b-9 was associated with poor kidney allograft survival.⁷ Nevertheless, despite the major role that C5b-9 plays in the glomerular damage of MN, its prognostic impact has never been studied.

This study aimed to determine the frequency and location of C5b-9 deposits in a well-phenotyped cohort of patients with pMN or sMN and to evaluate their impact on renal survival and remission rate.

METHODS

Patients and Samples

We retrospectively selected patients with MN from the databases of the Department of Pathology of Montpellier University Hospital. To be included, patients had to have undergone a kidney biopsy confirming a diagnosis of MN from December 2004 to December 2015. Patient biopsies were analyzed by light microscopy and immunofluorescence with anti-IgA, anti-IgG, anti-IgM, anti-C3, anti-C1q, anti-Kappa, and anti-Lambda staining. pMN was diagnosed in the absence of sMN features such as positivity for antinuclear antibodies; a history of syphilis, HIV, hepatitis B, or hepatitis C infection; cancer; or other immune pathologies (cryoglobulinemia, sarcoidosis, graft-versus-host-disease, etc.).

Exclusion criteria were as follows: pediatric patients (i.e., under 18 years of age), renal transplant patients, patients lost to follow-up or for whom the medical charts could not be retrieved, insufficient renal tissue sample for further immunohistochemistry (i.e., <2 nonsclerosed glomeruli in each recut section), patients with MN associated with another nephropathy on biopsy, and patients with an uncertain diagnosis of MN. All patients provided written informed consent to participate.

Immunohistochemical Staining for PLA2R1, C4d, and C5b-9

Staining for phospholipase A2 receptor 1 (PLA2R1), C4d, and C5b-9 was performed for all biopsies by immunohistochemistry. Briefly, paraffin-embedded sections were cut at a thickness of 3 μ m, deparaffinized and subjected to antigen retrieval. After blocking endogenous peroxidases, the sections were incubated with the relevant primary antibody. Binding of the primary antibody was visualized using the appropriate horseradish peroxidase-labeled secondary antibody and diaminobenzidine as the chromogen. Finally, the sections were counterstained with hematoxylin. A biopsy from a native kidney (kidney unsuitable for

transplantation because of vascular issues) was used as negative control.

The primary antibodies included the following: (i) rabbit polyclonal antibodies antihuman PLA2R1 (HPA012657, 1/100 dilution, Sigma-Aldrich); (ii) rabbit monoclonal antibodies antihuman C4d (DB107, clone A24-T, dilution 1/100; DB Biotech); and (iii) mouse monoclonal antibodies antihuman C9 neopeptide (clone B7, dilution 1/50000; gift from Paul Morgan, Cardiff, United Kingdom), which is highly specific to C5b-9 fixation in the membrane. C9 neopeptide detection was enhanced by the EnVision FLEX kit with linker (Dako).

The optimum antibody dilution and incubation for antihuman C9 neopeptide antibodies was determined empirically by performing a titration experiment and serial dilutions on positive (class IV lupus nephritis) and negative controls.

The stained sections were evaluated by 2 renal pathologists who were blinded to the clinical and biological data. The staining intensity was evaluated using a semiquantitative scoring system (negative = 0, weak = +, moderate = ++, and strong = +++). Staining was not studied on glomerular scars.

Study End Points

The primary end point was renal survival, which was calculated from the date of kidney biopsy to the date of renal failure (defined as progression to a glomerular filtration rate (GFR) <30 ml/min per 1.73 m² calculated by the Chronic Kidney Disease - Epidemiology Collaboration [CKD-EPI] formula).

Secondary end points were clinical remission rates (partial or complete) of nephrotic syndrome at 6 months and 12 months after diagnosis and at the end of follow-up. Complete or partial remission were defined according to the 2012 Kidney Disease: Improving Global Outcomes guidelines.⁸ Complete remission was defined as a urinary protein-to-creatinine ratio <0.3 g/g, accompanied by a normal serum albumin concentration and preserved kidney function (GFR >30 ml/min per 1.73 m² calculated by the CKD-EPI formula). Partial remission was defined as a urinary protein-to-creatinine ratio <3.5 g/g with >50% reduction of proteinuria, accompanied by an improvement or normalization of the serum albumin concentration and preserved kidney function. Cases that did not meet these definitions were considered as treatment failures.

Statistical Analysis

Continuous variables were described as means (\pm SD) or medians [25th and 75th percentile] according to their distribution. Categorical variables were represented as counts and proportions. The *t* test, Wilcoxon test, and χ^2 and Fisher exact tests were used to compare the

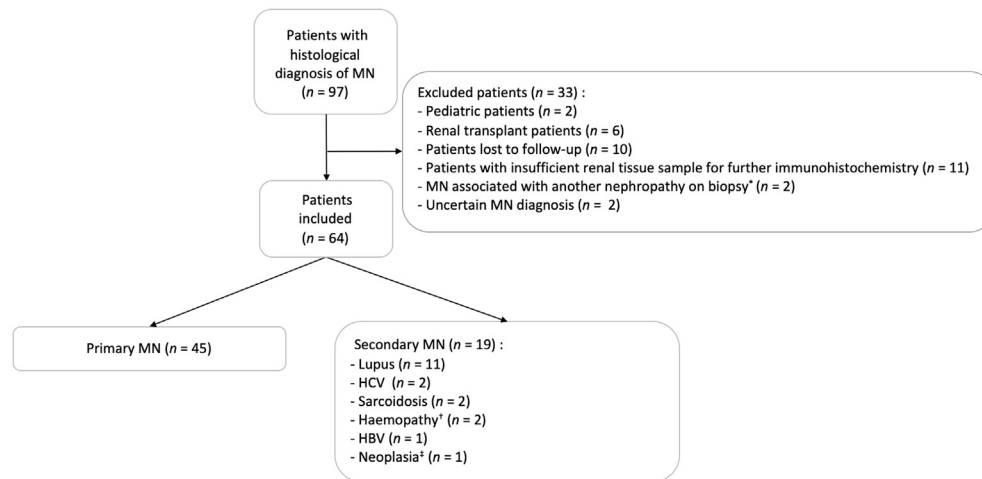


Figure 1. Flowchart. HBV, hepatitis B virus; HCV, hepatitis C virus; IHC, immunochemistry; MN, membranous nephropathy. *One extracapillary glomerulonephritis with antineutrophil cytoplasmic antibodies and one diabetic nephropathy. †Waldenström's disease and allograft hematopoietic stem cell transplantation with graft-versus-host disease. ‡Thyroid neoplasia.

groups as appropriate. Survival curves were assessed by the Kaplan–Meier method and compared among the groups with the log-rank test. Cox proportional hazards models were used to estimate the hazard ratios, 95% confidence intervals (CIs), and *P* values for (i) renal failure and (ii) remission of nephrotic syndrome. According to the sample size, we selected our explanatory variables from a set of sensible clinical, biological, immunologic, and histologic parameters; domain expert knowledge and preliminary principal component analysis performed leaving end points out helped us select a few relevant predictor variables that we used in our multivariable Cox survival models for renal failure and for remission. During the modeling stage, we choose to consider only linear effects between predictor and outcome variables, and we did not consider any interaction effects between the predictor variables. The resulting multivariable survival models obtained on the full set of selected variables (full models) resulted in unbiased CI and *P* values for the predictors. Results of univariate Cox models on the full set of candidate variables have also been performed for comparison. Prism software (version 8.4.3; GraphPad Software Inc., San Diego, CA) and R software (version 3.6.3; R Foundation for Statistical Computing, Vienna, Austria) were used to perform all analyses. All tests were 2-sided, and *P* values <0.05 were considered statistically significant.

RESULTS

Patient Characteristics

During the study period, 97 patients were diagnosed with MN at Montpellier University Hospital. Among them, 33 were excluded for the following reasons: pediatric patients (*n* = 2), renal transplant patients (*n* = 6),

patients lost to follow-up or for whom the medical charts could not be retrieved (*n* = 10), patients for whom there was no histologic material available to perform complementary staining (*n* = 11), patients with MN associated with another nephropathy on biopsy (*n* = 2), and patients with an uncertain diagnosis of MN (*n* = 2). Finally, 64 patients (45 with pMN and 19 with sMN) were included (Figure 1), with a median follow-up of 94.5 months (interquartile range, 59.25–132.5 months). Their characteristics are summarized in Table 1.

The patients were mostly men (59.4%), and the median age was 54 years (interquartile range, 36–68 years). At the time of kidney biopsy, the median creatinine level was 93.0 $\mu\text{mol/l}$ (interquartile range, 67.2–132.8 $\mu\text{mol/l}$), the median GFR (CKD-EPI formula) was 73.7 ml/min per 1.73 m^2 (interquartile range, 46.6–106.4 ml/min per 1.73 m^2), the median proteinuria was 5.5 g/g (interquartile range, 3.0–9.0 g/g), and the median albuminemia was 2.7 g/dl (interquartile range, 2.2–3.2 g/dl). Forty-two patients (65.6%) had nephrotic syndrome at the time of kidney biopsy.

At diagnosis, 7 patients (5 pMN and 2 sMN) were tested for anti-PLA2R1 antibodies in the serum. Of these, 2 (28.5%) were positive, both of whom had pMN.

All but 1 patient received renin-angiotensin-aldosterone system inhibitors. Forty-five patients (70.3%) received at least 1 immunosuppressive therapy during follow-up. The first-line immunosuppressive treatments used were exclusive corticosteroid therapy (in 16 patients, 25.0%), cyclosporine (in 7 patients, 10.9%), cyclosporine plus corticosteroid (in 9 patients, 14.1%), mycophenolate mofetil plus corticosteroid (in 4 patients, 6.3%), azathioprine plus corticosteroid (in 1 patient, 1.6%), alkylating agent plus corticosteroid (in 4 patients, 6.3%), and rituximab (in 4 patients, 6.3%). Two patients (3.1%) also received antiviral therapy for hepatitis C infection.

Table 1. Patient characteristics at baseline and during follow-up

Variable	All patients, n = 64	Primary MN, n = 45	Secondary MN, n = 19	P value
Age (yr), median (Q1–Q3)	54 (36–68)	60 (45–69)	36 (24–49)	0.0002
Male, n (%)	38 (59.4)	32 (71.1)	6 (31.6)	0.003
Diagnostic delay (mo), median (Q1–Q3)	5.0 (1.0–8.0)	5.0 (1.8–8.0)	6.0 (1.0–7.5)	0.9
Medical history				
Hypertension, n (%)	21 (32.8)	19 (42.3)	2 (10.5)	0.01
Obesity, n (%)	11 (17.2)	8 (17.8)	3 (15.8)	0.8
Diabetes, n (%)	6 (9.4)	6 (13.3)	0 (0)	0.1
Active smoking or stopped for less than 3 years, n (%)	12 (18.7)	8 (17.8)	4 (21)	0.8
Biology at the time of kidney biopsy				
Creatininemia ($\mu\text{mol/l}$), median (Q1–Q3)	93.0 (67.2–132.8)	106.0 (85.0–137.0)	64.0 (54.5–83.5)	0.002
GFR (ml/min per 1.73 m ²), median (Q1–Q3)	73.7 (46.6–106.4)	59.8 (45.2–83.7)	108.6 (82.8–126.5)	0.002
Urinary protein-to-creatinine ratio (g/g), median (Q1–Q3)	5.5 (3.0–9.0)	6.8 (3.7–9.7)	3.5 (2.1–6.0)	0.02
Albuminemia (g/dl), median (Q1–Q3)	2.7 (2.2–3.2)	2.5 (2.1–3.1)	2.8 (2.3–3.4)	0.3
Hematuria, n (%)	31 (48.4)	22 (48.9)	9 (47.4)	0.9
Treatment				
RAAS inhibitors, n (%)	63 (98.4)	45 (100.0)	18 (94.7)	0.3
Immunosuppressive treatment ^a , n (%)	45 (70.3)	30 (66.7)	15 (78.9)	0.3
Outcome after kidney biopsy				
Remission 6 months after kidney biopsy, n (%)	19 (29.7)	12 (26.7)	7 (36.8)	0.4
Remission 12 months after kidney biopsy, n (%)	34 (53.1)	22 (48.9)	12 (63.2)	0.3
Remission at last follow-up, n (%)	45 (70.3)	31 (68.9)	14 (73.8)	0.7
Renal failure ^b 5 years after kidney biopsy, n (%)	14 (21.9)	11 (24.4)	3 (15.8)	0.4
Renal failure ^b at last follow-up, n (%)	19 (29.7)	16 (35.5)	3 (15.8)	0.1
Death, n (%)	3 (4.7)	2 (4.4)	1 (5.3)	1
Follow-up (mo), median (Q1–Q3)	94.5 (59.2–132.5)	90.0 (54.0–132.0)	104.0 (70.0–133.0)	0.4

GFR, glomerular filtration rate; MN, membranous nephropathy; Q1, first quartile 1; Q3, third quartile; RAAS, renin-angiotensin-aldosterone system.

^aThe immunosuppressive treatments used were as follows: exclusive corticosteroid, cyclosporine, cyclosporine plus corticosteroid, mycophenolate mofetil plus corticosteroid, azathioprine plus corticosteroid, alkylating agent plus corticosteroid, and rituximab.

^bRenal failure was defined by progression to a GFR <30 ml/min per 1.73 m² estimated by the CKD-EPI formula.

The histologic parameters are listed in Table 2. Stage I was the most common MN pathologic stage (45.3%). Few chronic lesions were observed on the biopsies: the median percentage of glomerular scars was 2.9% (interquartile range, 0.0–12.5); 21 patients (32.8%) had moderate to severe chronic vascular lesions (defined as

fibrous obstruction of the lumen of arterioles or medium-caliber vessels >25%), and the median cortical surface with interstitial fibrosis and tubular atrophy was 10% (interquartile range, 0–10).

PLA2R1 staining was positive in the glomeruli of 44 patients (68.8%), including 40 (90.9%) with pMN.

Table 2. Histologic parameters at baseline

Variable	All patients, n = 64	Primary MN, n = 45	Secondary MN, n = 19	P value
MN pathologic stage				
I, n (%)	29 (45.3)	19 (42.3)	10 (52.6)	0.4
II, n (%)	16 (25.0)	10 (22.2)	6 (31.6)	0.4
III, n (%)	19 (29.7)	16 (35.5)	3 (15.8)	0.1
IV, n (%)	0 (0.0)	0 (0.0)	0 (0.0)	1
Glomerular scars, (%), median (Q1–Q3)	2.9 (0.0–12.5)	4.3 (0.0–16.3)	0 (0.0–6.6)	0.1
Interstitial fibrosis and tubular atrophy (%), median (Q1–Q3)	10 (0–10)	10 (0–25)	5 (0–23)	0.2
Moderate to severe chronic vascular lesions ^a , n (%)	21 (32.8)	18 (40)	3 (15.8)	0.08
Immune deposits				
IgG, n (%)	64 (100.0)	45 (100.0)	19 (100.0)	1
IgA, n (%)	27 (42.2)	12 (26.7)	15 (78.9)	0.0002
IgM, n (%)	36 (56.2)	22 (48.9)	14 (73.7)	0.07
C3, n (%)	61 (95.3)	43 (95.6)	18 (94.7)	1
C1q, n (%)	23 (35.9)	9 (20.0)	14 (73.7)	<0.0001
C4d, n (%)	61 (95.3)	43 (95.6)	18 (94.7)	1
PLA2R1, n (%)	44 (68.8)	40 (88.9)	4 (21.0)	<0.0001
C5b-9, n (%)	29 (45.3)	23 (51.1)	6 (31.6)	0.2

IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; MN, membranous nephropathy; PLA2R1, phospholipase A2 receptor type 1; Q1, first quartile 1; Q3, third quartile.

^aModerate to severe vascular lesions were defined by an intimal fibrosis obstructing more than 25% of the vascular lumen of arterioles and/or medium-caliber vessels.

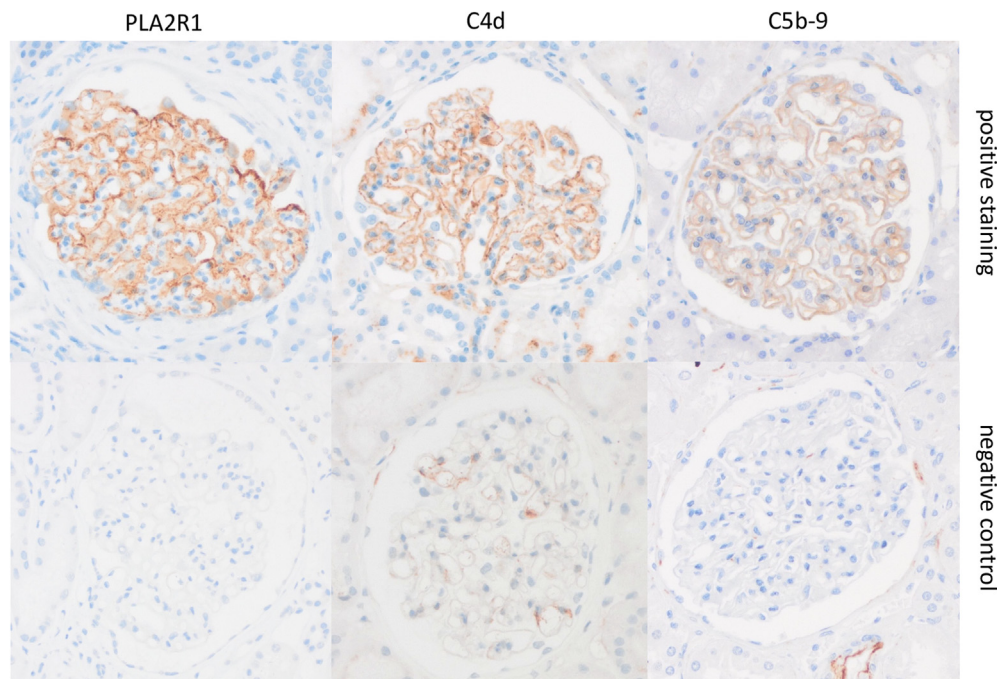


Figure 2. *PLA2R1*, C4d, and C5b-9 deposits by immunohistochemistry on renal biopsies of membranous nephropathy and on negative control ($\times 400$). *PLA2R1*, phospholipase A2 receptor type 1.

There were 4 cases of sMN with positive *PLA2R1* staining corresponding to 2 MN secondary to sarcoidosis, 1 MN secondary to hepatitis C infection, and 1 MN secondary to hepatitis B infection. The glomerular *PLA2R1* deposits were granular and located in the subepithelial space (Figure 2). Of the 5 patients with pMN who had serum *PLA2R1* antibody testing at diagnosis, the 2 who had positive serum antibodies also had positive *PLA2R1* staining, and of the 3 patients without serum *PLA2R1* antibodies, 2 also had negative *PLA2R1* staining, and only 1 had positive *PLA2R1* staining.

There was no discordance between the 2 renal pathologists regarding the positivity of the staining. There were no significant *PLA2R1* staining on the normal kidney biopsy (Figure 2).

Frequency of Complement Deposition on Renal Biopsies

The findings are summarized in Table 2. C4d was positive in the glomeruli of 61 patients (95.3%). The glomerular C4d deposits were granular and located in the subepithelial space (Figure 2). The C4d staining intensity was as follows: weak (+) in 15 patients (24.6%), moderate (++) in 28 (45.9%), and strong (+++) in 18 (29.5%). The staining was diffuse and global for all the patients.

C5b-9 was positive in the glomeruli of 29 patients (45.3%). The glomerular C5b-9 deposits were granular and located in the subepithelial space (Figure 2). All biopsies with glomerular C5b-9 deposits also had C4d

and C3 deposits. The C5b-9 staining intensity was as follows: weak (+) in 17 patients (58.6%), moderate (++) in 9 patients (31.1%), and strong (+++) in 3 patients (10.3%). The staining was diffuse and global for all the patients. There was no discordance between the 2 renal pathologists regarding the positivity of the staining.

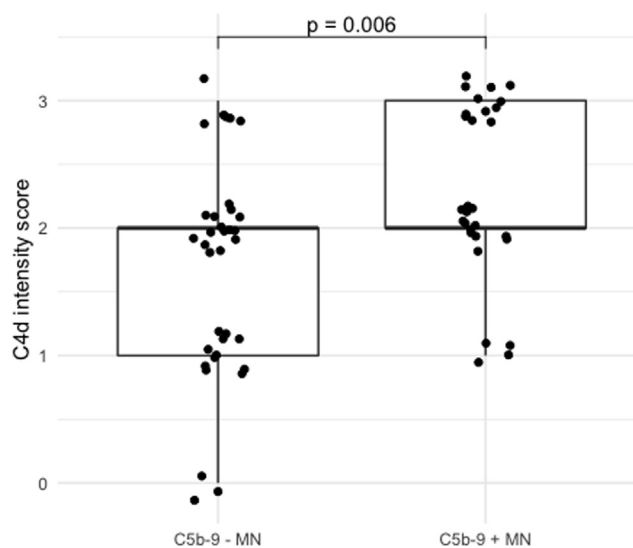


Figure 3. C4d staining intensity score according to glomerular staining for C5b-9. C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 – MN, membranous nephropathy without glomerular C5b-9 deposits; MN, membranous nephropathy.

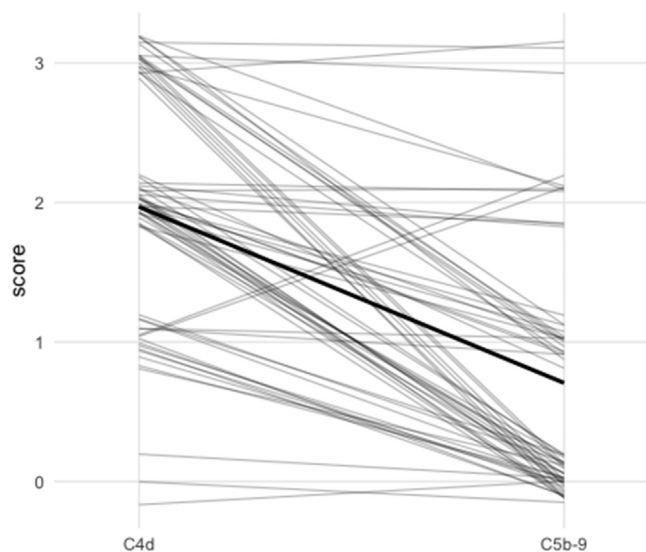


Figure 4. C4d versus C5b-9 staining intensity scores for the study patients. The thin lines represent a relationship between the 2 scores for a given patient; jitter has been applied to the scores to easily differentiate the patients. The broad line defines the average score for all patients.

The intensity of C4d staining was associated with positive C5b-9 staining ($P = 0.006$) (Figure 3). We compared C4d staining intensity with C5b-9 staining intensity for each patient (Figure 4) and observed the

following: (i) all the patients with glomerular C5b-9 deposits had glomerular C4d deposits, and (ii) the average intensity of C5b-9 staining was weaker than the average intensity of C4d staining.

There were no significant C4d and C5b-9 staining on the negative control kidney biopsy (Figure 2). Because kidney biopsy samples were collected over an extended time period, we analyzed the time between kidney biopsy and C5b-9 staining to ensure that it did not affect sample positivity or negativity. We found no significant difference in the distribution of C5b-9 staining positivity or negativity according to the time of diagnosis (data not shown).

Characteristics of Patients With Positive Glomerular C5b-9 Deposits

Comparison of the characteristics of patients with glomerular C5b-9 deposits (C5b-9 + MN) and patients without glomerular C5b-9 deposits (C5b-9 – MN) is detailed in Tables 3 and 4.

There was no statistically significant difference between the 2 groups in terms of sex ratio, age, proportion of sMN, and time to diagnosis (Table 3). Compared with C5b-9 – MN patients, C5b-9 + MN patients had higher creatinemia (113.0 vs. 77.0 $\mu\text{mol/l}$, $P = 0.0009$), higher proteinuria (8.0 vs. 4.1 g/g, $P = 0.001$),

Table 3. Patient characteristics at baseline according to glomerular C5b-9 staining

Variable	C5b-9 + MN, n = 29	C5b-9 – MN, n = 35	P value
Age (yr), median (Q1–Q3)	55.0 (35.0–64.0)	50.0 (38.5–68.5)	0.8
Male, n (%)	21 (72.4)	17 (48.6)	0.05
Secondary MN, n (%)	6 (20.7)	13 (37.1)	0.2
Lupus	5 (17.2)	6 (17.1)	1
HCV	0 (0.0)	2 (5.7)	0.2
Sarcoidosis	1 (3.5)	1 (2.9)	0.9
Hemopathy	0 (0.0)	2 (5.7)	0.2
HBV	0 (0.0)	1 (2.9)	0.4
Neoplasia	0 (0.0)	1 (2.9)	0.4
Diagnostic delay (mo), median (Q1–Q3)	5.0 (2.0–9.0)	5.5 (1.0–7.7)	1
Medical history			
Hypertension, n (%)	12 (41.4)	9 (25.7)	0.2
Obesity, n (%)	4 (13.8)	7 (20.0)	0.5
Diabetes, n (%)	4 (13.8)	2 (5.7)	0.3
Active smoking or stopped for less than 3 years, n (%)	9 (31.0)	3 (8.6)	0.02
Biology at the time of kidney biopsy			
Creatininemia ($\mu\text{mol/l}$), median (Q1–Q3)	113.0 (89.0–164.0)	77.0 (61.0–108.5)	0.0009
GFR (ml/min per 1.73 m ²), median (Q1–Q3)	58.5 (38.9–83.7)	87.3 (58.3–109.2)	0.009
Urinary protein-to-creatinine ratio (g/g), median (Q1–Q3)	8.0 (4.6–10.0)	4.1 (2.0–6.5)	0.001
Albuminemia (g/dl), median (Q1–Q3)	24 (22–30)	29 (23–34)	0.2
Hematuria, n (%)	17 (58.6)	14 (40.0)	0.1
Treatment			
RAAS inhibitors, n (%)	29 (100.0)	34 (97.1)	1
Immunosuppressive treatment ^a , n (%)	22 (75.9)	23 (65.7)	0.4
Follow-up (mo), median (Q1–Q3)	96 (54–134)	93 (60–132)	0.5

C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 – MN, membranous nephropathy without glomerular C5b-9 deposits; GFR, glomerular filtration rate; HBV, hepatitis B virus; HCV, hepatitis C virus; MN, membranous nephropathy; Q1, first quartile 1; Q3, third quartile; RAAS, renin-angiotensin-aldosterone system.

^aThe immunosuppressive treatments used were exclusive corticosteroid, cyclosporine, cyclosporine plus corticosteroid, mycophenolate mofetil plus corticosteroid, azathioprine plus corticosteroid, alkylating agent plus corticosteroid, and rituximab.

Table 4. Histologic parameters at baseline according to glomerular C5b-9 staining

Variable	C5b-9 + MN, n = 29	C5b-9 - MN, n = 35	P value
MN pathologic stage			
I, n (%)	11 (38.0)	18 (51.5)	0.3
II, n (%)	7 (24.0)	9 (25.7)	0.9
III, n (%)	11 (38.0)	8 (22.8)	0.2
IV, n (%)	0 (0.0)	0 (0.0)	1
Glomerular scars (%), median (Q1–Q3)	4.7 (0.0–16.9)	0.0 (0.0–12.5)	0.3
Interstitial fibrosis and tubular atrophy (%), median (Q1–Q3)	10 (3–15)	5 (0–10)	0.06
Moderate to severe chronic vascular lesions ^a , n (%)	10 (34.5)	11 (31.4)	0.8
Immune deposits			
IgG, n (%)	29 (100.0)	35 (100.0)	1
IgA, n (%)	14 (48.3)	13 (37.1)	0.4
IgM, n (%)	14 (48.3)	22 (62.8)	0.2
C3, n (%)	29 (100.0)	32 (91.4)	0.1
C1q, n (%)	12 (41.4)	11 (31.4)	0.4
C4d, n (%)	29 (100.0)	32 (91.4)	0.1
PLA2R1, n (%)	23 (79.3)	21 (60.0)	0.1

C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 - MN, membranous nephropathy without glomerular C5b-9 deposits; IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; MN, membranous nephropathy; PLA2R1, phospholipase A2 receptor type 1; Q1, first quartile 1; Q3, third quartile.

^aModerate to severe vascular lesions were defined by an intimal fibrosis obstructing more than 25% of the vascular lumen of arterioles and/or medium-caliber vessels.

and lower GFR (58.5 vs. 87.3 ml/min per 1.73 m², $P = 0.009$), without any difference in the treatments administered (Table 3). There were no differences in the histologic characteristics between the 2 groups (Table 4), in particular for pathologic stage and chronic fibrous lesions (i.e., glomerular scars, moderate to severe chronic vascular lesions, and interstitial fibrosis and tubular atrophy).

Remission Rate After MN Diagnosis According to the Glomerular Deposition of C5b-9

Remission rates in patients with glomerular deposition of C5b-9 were 10.3%, 34.5%, and 41.4% at 6 months, 12 months, and at the end of follow-up, respectively, compared with 45.7% ($P = 0.002$), 68.6% ($P = 0.007$),

and 85.7% ($P = 0.0002$), respectively, in patients without glomerular deposition of C5b-9 (Table 5).

Kaplan-Meier analysis demonstrated a faster time to remission in patients without glomerular deposition of C5b-9 ($P = 0.0005$ by log-rank test), with a median time to remission of 18 months in patients with glomerular deposition of C5b-9 compared with 9 months in patients without glomerular deposition of C5b-9 (Figure 5).

Of the 45 patients receiving immunosuppressive therapy during follow-up, 26 (57.8%) achieved remission after the first-line treatment, including 10 (38.5%) with glomerular deposition of C5b-9, and 16 (61.5%) without. Nineteen patients (42.2%) were in therapeutic failure and required additional treatments, including 12 (63.2%) with glomerular deposition of C5b-9, and 7 (36.8%) without ($P = 0.1$).

Table 5. Patient outcomes according to glomerular C5b-9 staining

Variable	C5b-9 + MN, n = 29	C5b-9 - MN, n = 35	P value
Remission 6 months after kidney biopsy			
Complete remission, n (%)	0 (0.0)	8 (22.8)	0.006
Partial remission, n (%)	3 (10.3)	8 (22.8)	0.2
Remission (partial or complete), n (%)	3 (10.3)	16 (45.7)	0.002
Remission 12 months after kidney biopsy			
Complete remission, n (%)	3 (10.3)	16 (45.7)	0.002
Partial remission, n (%)	7 (24.0)	8 (22.8)	0.9
Remission (partial or complete), n (%)	10 (34.5)	24 (68.6)	0.007
Remission at last follow-up			
Complete remission, n (%)	7 (24.0)	23 (65.7)	0.0009
Partial remission, n (%)	5 (17.2)	7 (20.0)	0.3
Remission (partial or complete), n (%)	12 (41.4)	30 (85.7)	0.0002
Renal failure ^a 5 years after kidney biopsy, n (%)	10 (34.5)	4 (11.4)	0.03
Renal failure ^a at last follow-up, n (%)	15 (51.7)	4 (11.4)	0.0004
Dialysis or preemptive kidney transplant, n (%)	10 (34.5)	3 (8.6)	0.01
Death, n (%)	1 (3.5)	2 (5.7)	1

C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 - MN, membranous nephropathy without glomerular C5b-9 deposits.

^aRenal failure was defined by progression to a GFR < 30 ml/min per 1.73 m² estimated by the CKD-EPI formula.

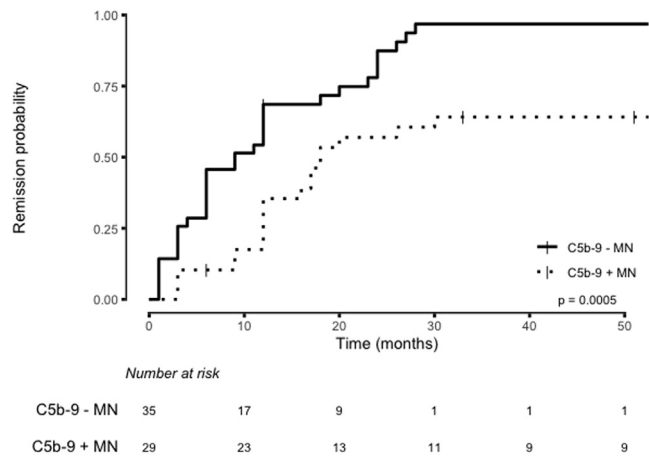


Figure 5. Time from kidney biopsy to remission according to glomerular staining for C5b-9. C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 – MN, membranous nephropathy without glomerular C5b-9 deposits; MN, membranous nephropathy.

Of the 19 patients who did not receive immunosuppressive therapy during follow-up, 16 (84.2%) achieved remission, including 4 (25.0%) with glomerular deposition of C5b-9, and 12 (75.0%) without. Three patients (15.8%), all with glomerular deposition of C5b-9, did not achieve remission and progressed to end-stage renal disease ($P = 0.01$).

Results of univariate Cox regression analyses are provided in Table 6. Multivariable Cox regression analysis was performed using the following 5

predictors: (i) glomerular deposition of C5b-9 (presence vs. absence), (ii) GFR (continuous), (iii) urinary protein-to-creatinine ratio (continuous), (iv) etiology of MN (primary vs. secondary), and (v) use of immunosuppressive treatment within 6 months of kidney biopsy (yes vs. no). In this multivariable model, the glomerular deposition of C5b-9 was associated with a lower remission rate, even after correcting for the GFR and urinary protein-to-creatinine ratio at baseline. The hazard ratio for remission of nephrotic syndrome was 0.48 in patients with glomerular deposition of C5b-9 (95% CI, 0.26–0.90; $P = 0.02$) (Table 6).

Renal Survival After MN Diagnosis According to the Glomerular Deposition of C5b-9

After a median follow-up of 94.5 months (range, 59.25–132.5 months), 19 patients (29.7%) had progressed to renal failure (progression to a GFR <30 ml/min per 1.73 m² calculated by the CKD-EPI formula): 51.7% (15 of 29) in the patients with glomerular deposition of C5b-9 and 11.4% (4 of 35) in the patients without ($P = 0.0004$) (Table 5). Renal survival over time according to the glomerular deposition of C5b-9 is represented in Figure 6. Renal survival was statistically lower among patients with glomerular deposition of C5b-9 ($P = 0.0006$ by log-rank test).

Results of univariate Cox regression analyses are provided in Table 7. Multivariable Cox regression analysis was performed using the following 5

Table 6. Univariate and multivariable analysis of variables associated with clinical remission

Variables	Univariate analysis			Multivariable analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age (yr)	1.00	0.99–1.02	0.9			
Male	0.71	0.41–1.22	0.2			
Secondary MN	1.44	0.80–2.60	0.2	1.02	0.52–1.98	1
Medical history						
Presence of high blood pressure before diagnosis	0.83	0.46–1.48	0.5			
Presence of obesity	0.75	0.36–1.53	0.4			
Presence of diabetes	0.65	0.26–1.62	0.4			
Presence of active smoking or stopped for less than 3 years	2.19	1.03–4.65	0.04			
Biology at the time of kidney biopsy						
Creatininemia (μmol/l)	0.99	0.98–0.996	0.001			
GFR (ml/min per 1.73 m ²)	1.01	1.01–1.02	0.002	1.01	0.999–1.02	0.09
Urinary protein-to-creatinine ratio (g/g)	0.98	0.93–1.03	0.4	1.001	0.95–1.06	0.96
Albuminemia (g/dl)	0.997	0.96–1.04	0.9			
Histology						
Percentage of glomerular scars	0.98	0.96–1.00	0.09			
Percentage of interstitial fibrosis and tubular atrophy	0.98	0.95–1.01	0.1			
Presence of moderate to severe chronic vascular lesions ^a	0.87	0.49–1.55	0.6			
Presence of C5b-9 in deposits	0.38	0.21–0.67	0.001	0.48	0.26–0.90	0.02
Treatment						
Use of immunosuppressive treatment ^b	1.02	0.58–1.78	0.9	1.14	0.64–2.04	0.7

CI, confidence interval; GFR, glomerular filtration rate; HR, hazard ratio; MN, membranous nephropathy.

^aModerate to severe vascular lesions were defined by an intimal fibrosis obstructing more than 25% of the vascular lumen of arterioles and/or medium-caliber vessels.

^bThe immunosuppressive treatments used were exclusive corticosteroid, cyclosporine, cyclosporine plus corticosteroid, mycophenolate mofetil plus corticosteroid, azathioprine plus corticosteroid, alkylating agent plus corticosteroid, and rituximab.

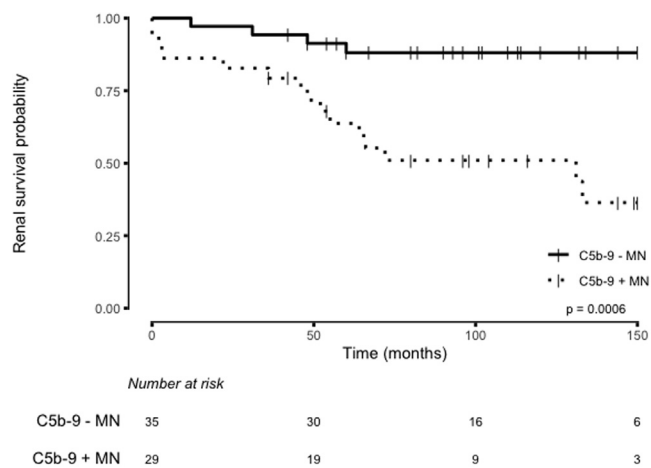


Figure 6. Renal survival over time after kidney biopsy according to glomerular staining for C5b-9. C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 – MN, membranous nephropathy without glomerular C5b-9 deposits; MN, membranous nephropathy.

predictors: (i) glomerular deposition of C5b-9 (presence vs. absence), (ii) GFR (continuous), (iii) clinical remission at month 6 (presence vs. absence), (iv) urinary protein-to-creatinine ratio (continuous), and (v) moderate to severe chronic vascular lesions (presence vs. absence). In this multivariable model, the glomerular deposition of C5b-9 was associated with renal failure, even after correcting for the GFR and urinary protein-

to-creatinine ratio at baseline. The baseline GFR (hazard ratio 0.97; 95% CI, 0.95–0.99; $P = 0.01$) and glomerular deposition of C5b-9 (hazard ratio 4.59; 95% CI, 1.41–14.89; $P = 0.01$) were statistically associated with renal failure (Table 7).

This last model was used to predict renal survival as a joint function of the GFR quartiles in the presence or absence of C5b-9 deposits, keeping constant the other predictors of the full model (patients achieving remission at month 6 and urinary protein-to-creatinine ratio fixed at its mean value). The effect of the presence of C5b-9 in deposits on renal survival is of the same magnitude as switching from the third to the first quartile of GFR. Presence of C5b-9 in deposits and altered GFR (first quartile) have a cumulative effect on renal survival (Figure 7).

DISCUSSION

To our knowledge, this is the first study to evaluate the impact of glomerular deposition of C5b-9 on renal survival and remission rates in patients with MN. In a cohort of 64 patients with pMN and sMN, we observed that glomerular C5b-9 deposits were present in 29 patients (45.3%). The C5b-9 deposits were systematically associated with C4d and C3 deposits. Complement deposition was not a binary event, and the intensity of C4d and C5b-9 staining was not uniform among

Table 7. Univariate and multivariable analysis of variables associated with renal survival

Variables	Univariate analysis			Multivariable analysis		
	HR	95% CI	P value	HR	95% CI	P value
Age (yr)	1.02	0.99–1.05	0.2			
Male	1.14	0.45–2.91	0.8			
Secondary MN	0.40	0.12–1.36	0.1			
Medical history						
Presence of high blood pressure before diagnosis	3.62	1.42–9.22	0.007			
Presence of obesity	0.77	0.22–2.78	0.7			
Presence of diabetes	1.96	0.57–6.74	0.3			
Presence of active smoking or stopped for less than 3 years	0.48	0.18–1.26	0.1			
Biology at the time of kidney biopsy						
Creatininemia ($\mu\text{mol/l}$)	1.02	1.01–1.03	<0.001			
GFR ($\text{ml/min per } 1.73 \text{ m}^2$)	0.97	0.95–0.99	<0.001	0.97	0.95–0.99	0.01
Urinary protein-to-creatinine ratio (g/g)	1.03	0.95–1.11	0.5	0.97	0.86–1.09	0.6
Albuminemia (g/dl)	0.97	0.92–1.03	0.4			
Histology						
Percentage of glomerular scars	1.04	1.02–1.06	0.001			
Percentage of interstitial fibrosis and tubular atrophy	1.06	1.02–1.10	0.001			
Presence of moderate to severe chronic vascular lesions ^a	3.33	1.29–8.60	0.01	2.73	0.99–7.53	0.05
Presence of C5b-9 in deposits	5.61	1.86–16.91	0.002	4.59	1.41–14.89	0.01
Outcome						
Remission at month-6	0.38	0.11–1.32	0.1	0.84	0.23–3.05	0.8
Treatment						
Use of immunosuppressive treatment ^b	1.1	0.40–3.06	0.9			

CI, confidence interval; HR, hazard ratio; GFR, glomerular filtration rate; MN, membranous nephropathy.

^aModerate to severe vascular lesions were defined by an intimal fibrosis obstructing more than 25% of the vascular lumen of arterioles and/or medium-caliber vessels.

^bThe immunosuppressive treatments used were exclusive corticosteroid, cyclosporine, cyclosporine plus corticosteroid, mycophenolate mofetil plus corticosteroid, azathioprine plus corticosteroid, alkylating agent plus corticosteroid, and rituximab.

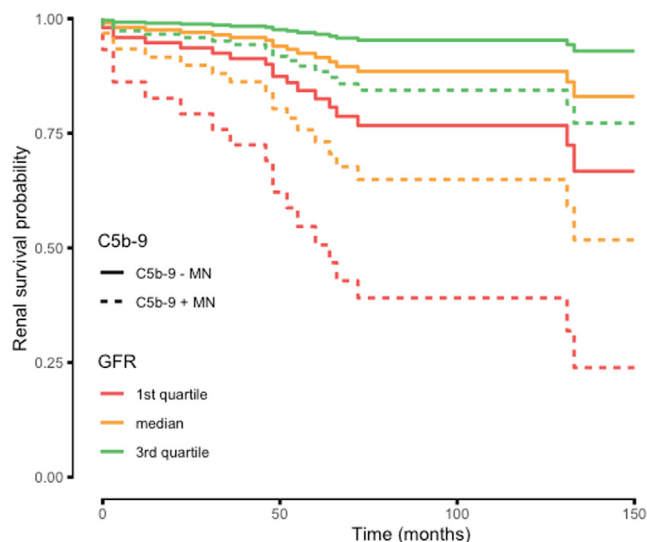


Figure 7. Renal survival over time after kidney biopsy according to GFR levels in the presence or absence of glomerular C5b-9 deposits. C5b-9 + MN, membranous nephropathy with glomerular C5b-9 deposits; C5b-9 – MN, membranous nephropathy without glomerular C5b-9 deposits; GFR, glomerular filtration rate; MN, membranous nephropathy.

patients; the more C4d deposits a patient had, the more likely they were to have C5b-9 deposits. Interestingly, patients with glomerular deposition of C5b-9 had a more severe nephrotic syndrome at diagnosis, as well as a worse outcome with more treatment failures and lower renal survival, than patients without glomerular deposition of C5b-9.

The complement system plays an important role in the pathophysiology of MN.^{9,10} However, at present, the exact pathophysiologic mechanisms of MN are still unclear. In this study, 95.3% of the patients had glomerular deposits of C3 and C4d. Activation of the complement is therefore almost systematic. However, glomerular deposition of C5b-9 was found in only 45.3% of patients. The use of a mouse monoclonal antihuman C9 neopeptide antibody makes our C5b-9 glomerular staining highly specific. Indeed, the membrane attack complex is a product of the assembly of the following 5 complement proteins: C5b, C6, C7, C8, and C9. The fusion of these proteins into C5b-9 leads to the expression of epitopes called “neopeptides,” which are not expressed on the native precursor proteins.¹¹

Several pathophysiologic hypotheses can be derived from these observations. In C5b-9 – MN, the complement system is partially regulated at the systemic and glomerular level—complement regulatory proteins CR1, DAF, MCP and CD59 are expressed by podocytes¹²—and the activation of the complement cascade is not complete. There is no formation of the membrane attack complex. Glomerular lesions in this group could therefore be secondary to other complement-mediated

mechanisms (e.g., modulation of the immune response, opsonization by C3b or proinflammatory role of C3a and C5a anaphylatoxins), and by mechanisms independent of complement and mediated directly by antibodies (e.g., direct cytotoxicity, modulation of intracellular cascades, inhibition or activation of the target antigen, etc.). This hypothesis is supported by several studies describing the direct effects of antibodies on molecular processes potentially involved in the pathogenesis of MN. In the Heymann nephritis model, antimegaline antibodies prevent the absorption of apolipoproteins by megaline, leading to the accumulation of apolipoproteins in glomerular immune deposits.¹³ The lipids of the apolipoproteins present in the glomerular deposits may then undergo peroxidation, causing damage to the glomerular basement membrane resulting in proteinuria.^{14,15} Furthermore, it has been shown in Heymann nephritis models that proteinuria can also develop in C6- and C3-deficient rats.^{16–18} In murine models of nephritis induced by passive transfer of antibodies to thrombospondin type-1 domain-containing 7A (*THSD7A*), proteinuria occurs without glomerular deposition of C3.^{19,20} In murine podocyte cell cultures, anti-*THSD7A* antibodies induce marked rearrangement of the cytoskeleton.¹⁹ In human podocyte cell cultures, *THSD7A* increases cell size, improves adhesion, reduces the detachment of type IV collagen-coated plaques, and decreases cell migration capacity.²¹ These observations suggest that *in vivo* *THSD7A* may be involved in the stabilization of podocytes and that anti-*THSD7A* antibodies may therefore structurally and functionally alter the permeability of the glomerular basement membrane by blocking *THSD7A* activity. The role of *PLA2R1* is less well known than that of *THSD7A*. Nevertheless, *in vitro* anti-*PLA2R1* antibodies interfere with the podocyte’s ability to bind to type IV collagen.²² These elements clearly demonstrate that podocyte injuries are the result of a complex multifactorial process, which requires further exploration.

In C5b-9 + MN, in addition to the mechanisms mentioned above, there is complete activation of the complement cascade—linked to overactivation of the complement system, a defect in the regulatory proteins or an imbalance between the activating and regulatory factors—leading to the formation of the membrane attack complex, which is itself responsible for podocyte injury. Overactivation of the complement system is highlighted in our study by the higher intensity of glomerular C4d deposition in C5b-9 + MN. The combination of all these mechanisms could lead to more severe and/or irreversible podocyte damage and explain why these patients have a more severe nephrotic syndrome and a poorer renal outcome.

Interestingly, studies have shown that high urinary C5b-9 levels correlate with poor clinical outcome in MN.^{23,24} High urinary C5b-9 levels could reflect the presence of severe glomerular inflammatory activity and thus correlate with glomerular C5b-9 deposits. However, we were unable to confirm this hypothesis because of the absence of available urine samples in our cohort.

This study opens up interesting therapeutic insights. Given the important role of complement in C5b-9 + MN, the use of anticomplement treatments is an attractive treatment option. Currently, only 1 study on complement inhibition in MN has been conducted.²⁵ The molecule used in this work was eculizumab, a monoclonal antibody that binds to C5 and prevents its cleavage, thereby limiting the generation of C5b-9. There was no statistically significant reduction in proteinuria in patients treated with eculizumab for 16 weeks compared with untreated patients. The inconsistent C5b-9 staining positivity that we have shown in our study may explain the lack of efficacy of eculizumab, which deserves to be re-evaluated in patients selected on the basis of C5b-9 glomerular staining. Because of the involvement of complement in the pathogenesis of many glomerulopathies, more anticomplement therapeutic agents are being developed, some in the setting of MN.²⁶ If anticomplement treatments are effective in these studies, patients with MN could benefit from personalized management, guided by the study of C5b-9 glomerular staining; patients with glomerular C5b-9 deposition may benefit from earlier and more intensive management, using anticomplement therapies in addition to prompt immunosuppressive therapy. Anticomplement treatments should be effective as long as the immunosuppressive treatment leads to a sufficient decrease in the level of circulating antibodies.

This work has several limitations. First, it was a retrospective, single-center study. Second, we excluded a significant number of patients who were lost to follow-up or who lacked sufficient histologic material. Third, this was a historical cohort receiving treatments that no longer reflect current practice and standard of care. These treatments are less effective and are associated with a higher risk of relapse than currently recommended treatments such as rituximab.²⁷⁻³¹ This may explain why the use of an immunosuppressive therapy was not an independent predictor of remission or renal survival in our multivariable analysis. However, the use of this historical cohort allowed us to have a long follow-up and to evaluate the interest of C5b-9 staining on long-term renal survival. We will validate the value of C5b-9 staining in a more recent cohort treated with

rituximab. Fourth, we included patients with sMN for whom the pathophysiology, treatment, and prognosis are different from patients with pMN. This was because we aimed to study the prognostic value of C5b-9 staining in MN independently of its etiology. Indeed, there is currently no reliable prognostic marker in sMN. Moreover, the patients with sMN included in this study had mainly lupus MN, in which complement plays an important physiopathological role. Finally, we were not able to study the staining of IgG subclasses because of a lack of histologic material, and we did not perform serum anti-PLA2R1 antibody testing for all patients because there was no test available in our center at the time of diagnosis for this cohort, and there was no serum bank.

In conclusion, we identified a new pattern of MN with complete activation of the complement cascade leading to the generation of the membrane attack complex and glomerular deposition of C5b-9. The presence of glomerular deposition of C5b-9 at diagnosis was associated with poor therapeutic response and poor renal survival. Other studies will be necessary in the future to validate the value of this histologic staining.

DISCLOSURE

MLQ reports grants from Alexion, Novartis, and Sanofi. All the other authors declared no competing interests.

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AUTHOR CONTRIBUTIONS

MT and MLQ designed the study; MT and HP interpreted biopsy staining; MT and MLQ wrote the manuscript; and all other authors contributed to the conduct of the study, recruited patients, and were involved in the review of results and final approval of the manuscript.

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