PATHOGENESIS AND MANAGEMENT OF GLOMERULAR DISEASES

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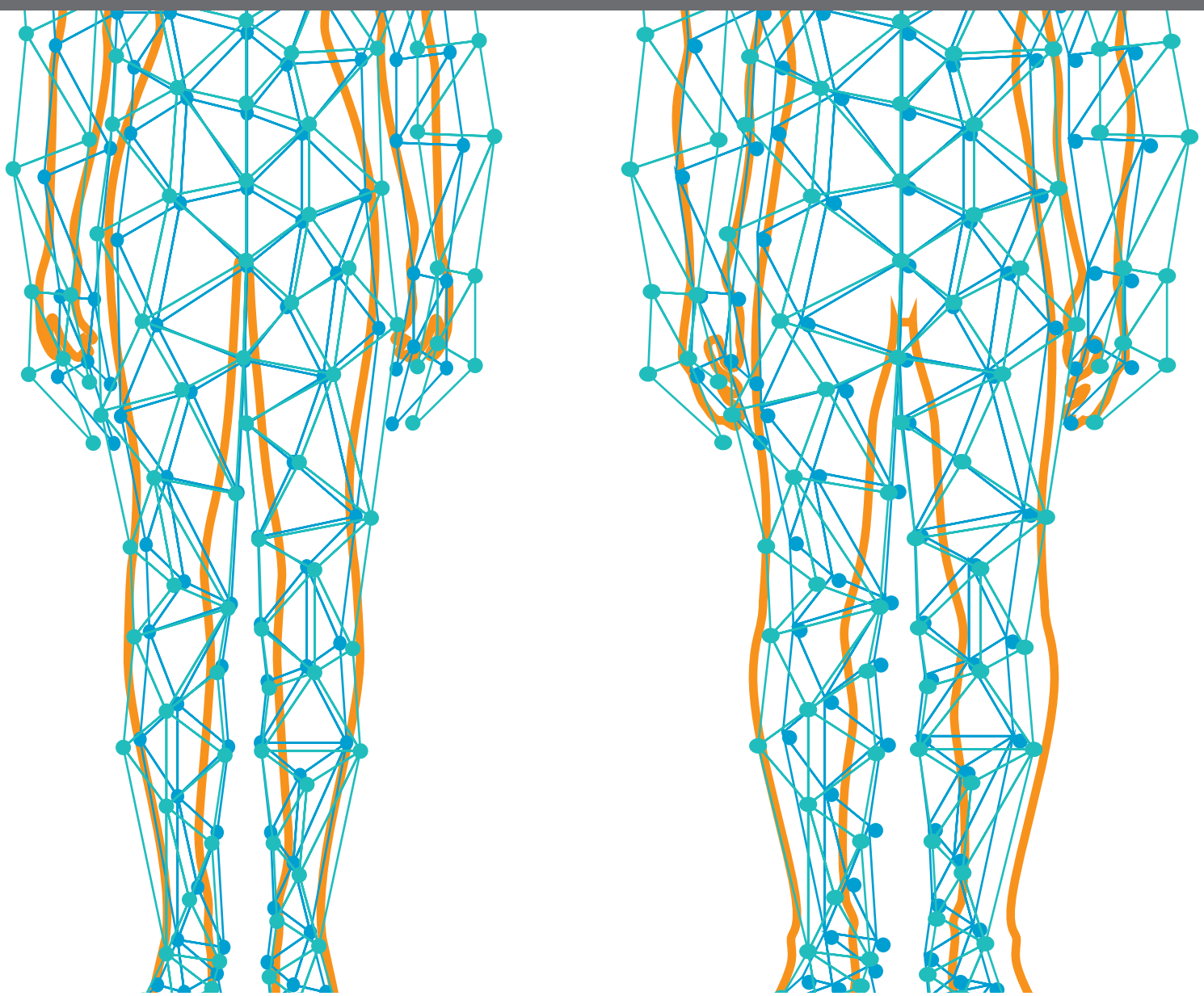

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PATHOGENESIS AND MANAGEMENT OF GLOMERULAR DISEASES

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Editorial: Pathogenesis and management of glomerular diseases

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Editorial on the Research Topic
Pathogenesis and management of glomerular diseases

Glomerular diseases are considered to be the result of inherited or acquired disorders and may manifest in a variety of clinical syndromes, including numerous pictures in terms of severity. A significant number of patients have no symptoms while others discover urinary abnormalities in routine screenings or they may experience low grade symptoms, such as macroscopic hematuria and edema in the lower extremities. Occasional patients present with rapidly progressive glomerulonephritis, a serious condition which may end up in advanced or end-stage kidney disease if remain untreated. Renal histopathology evaluation in combination with the characteristics of the clinical syndrome remains the cornerstone for accurate diagnosis and evidence-based treatment. During the past decade substantial progress has been made in this field, especially regarding the etiology and pathogenesis of these diseases. Recent knowledge has been added, including molecular mechanisms, genetic associations and immunologically-mediated forms of glomerulonephritis, underlining the autoimmune basis associated with genetic risk factors and environmental stimulus leading to immune-mediated injury of the glomeruli (1, 2). The role of animal models studies has been significant in this part, supporting a translational model of how immune reactions mediate the glomerular lesion, which is identified by renal pathologists in the biopsy specimen (1). It has been postulated and presented by several researchers that immune responses to infective agents and self-antigens are implicated in most forms of glomerulonephritis. Toll-like receptors and complement substances are considered to take part in these immune responses, while monocytes and resident glomerular cells are being activated initiating inflammatory reactions in the glomeruli (1). Several lesions in the glomerular filtration barrier are being developed, allowing passage of red blood cells and protein from the blood space (glomerular capillary lumen) into the urine. Therefore, microscopic hematuria, with or without proteinuria, is one of the most common clinical manifestations of glomerular diseases, including IgA nephropathy (IgAN), ANCA-vasculitis, and lupus nephritis (3–8). Clinicians with

long standing experience in the field of glomerular diseases regard persistent hematuria as a sign of continuing disease activity, although there are nephrologists who consider microscopic hematuria as a non-threatening finding, resulting from previous damage (8–10). However, the role of microhematuria in the development of glomerular diseases and progressive kidney damage lately received more consideration, since it was shown to be independently associated with progression of renal disease in patients with IgAN (11–13). Importantly, in this Research Topic of *Frontiers in Medicine,* He, Yu et al. report that a higher level of initial hematuria was a remarkable predictor of relapse in patients with primary membranous nephropathy (MN), while the degree and persistence of microscopic hematuria were independently associated with kidney disease progression. The vast majority of patients with primary MN (80%) present with nephrotic-range proteinuria, the remaining 20% have subnephrotic proteinuria while microhematuria appears in ∼50–60% of patients overall (14–19). As shown from this study, the hazards of kidney disease progression in patients with idiopathic MN and persistent hematuria was significantly higher than those with non-persistent hematuria during follow-up. As said above glomerular hematuria is caused by injuries in the glomerular filtration barrier structure. In patients with glomerular inflammation infiltrating inflammatory cells, such as leukocytes may release metalloproteinases and reactive oxygen species and the glomerular basement membrane becomes more susceptible to ruptures (4, 20, 21). On the other hand, persistent glomerular hematuria may stimulate renal damage *via* the oxidative stress caused by the release of hemoglobin and iron which is realized from broken erythrocytes into renal tubular cells (4, 10, 21–24). Yet, remission of hematuria was independently associated with reduced risk for renal progression (37%) compared with patients with ongoing microhematuria. Therefore, hematuria diminution may be a predictor of renal survival for patients with primary MN although its precise relationship with end-stage kidney disease or death and histolopathological findings requires to be elucidated in prospective studies.

The same group (He, Zha et al.) explored the issue of prognosis in patients with primary MN and subnephrotic proteinuria. As shown by previous investigators, although 20% of patients with primary MN present with subnephrotic range proteinuria, 61% of them later develop nephrotic range proteinuria, usually within the first year (14–19, 25). There is also long-term benefit of persistent subnephrotic proteinuria with renal survival of >80–90% at 10 years (26). More importantly, in practice, it is probable that these patients might not be properly observed and monitored, putting them at advanced risk of developing nephrotic range proteinuria and chronic kidney disease in the long term. In this regard, studying 205 cases with biopsy-proven primary MN and subnephrotic proteinuria, who had a minimum of 18 months of follow-up, the authors found that initial proteinuria was an independent predictor for partial remission, complete remission, and nephrotic proteinuria progression. The degree of the initial microhematuria was also associated with an increased risk of development of nephrotic range proteinuria. Hence, among patients with primary MN and subnephrotic proteinuria, although the overall prognosis was favorable, future detection and treatment of proteinuria plays a critical role.

The role of tertiary lymphoid organs (TLOs) in the pathogenesis of MN and its clinical associations with the disease, was explored by Wang Z-f et al. in 442 patents with biopsy proven MN. It was found that TLOs, which were defined as structures of accumulated lymphoid cells, were frequently discovered in the renal tissue in these patients. The TLOs density correlated with the severity of the clinical picture, i.e., the disease was worse with increasing TLOs. Patients who had TLOs were more likely to have anti-phospholipase A2 receptor autoantibodies in their circulation while patients without TLOs had significantly higher probability to achieve complete remission.

Lupus MN, a type of secondary form of this disease, accounting for 10–20% of total cases of lupus nephritis, is generally associated with a better patient and renal survival, compared to proliferative classes of renal involvement. In this issue of the journal (Kapsia et al.) a report of 27 patients with pure lupus MN, underlined the good prognosis, which is associated with type of lupus glomerulopathy. The patients were followed for a median period of 77 months, all had eGFR > 60 ml/min/1.73 m2 and long-term renal survival was very good.

As expected, the issue of prognosis remains crucial in all patients with glomerular disorders and particularly in IgAN because patients may present with various clinical and histopathological pictures, in terms of severity. In this regard, Dong et al. after studying 1,424 patients with IgAN found that arterial-arteriolar sclerosis was associated with the composite outcome of >50% reduction in estimated glomerular filtration rate, end-stage kidney disease or death and was an independent risk factor for the progression of IgAN. The severity of arterial-arteriolar sclerosis was associated with these outcomes and there was shown a trend that it might serve as an independent risk marker for progression of IgAN. Likewise, Wang Y-N et al. showed that the prevalence of hyperhomocysteinemia was high in patients with IgAN and greater than that seen in other forms of glomerular disease. The homocysteine/eGFR ratio was associated with the histopathologic features of IgAN, including the proportion of global glomerulosclerosis, the proportion of ischemia originated glomerular sclerosis, and the severity of tubular atrophy/interstitial fibrosis. Importantly, this ratio was an independent risk factor for chronic kidney disease progression event.

Finally, one study addressed an important consideration in individuals with glomerular disease, that of pregnancy. Women with glomerular disease may be at particular risk for adverse pregnancy outcomes and at risk for subfertility and

early menopause (27). Marinaki et al. describe outcomes in 22 women with glomerular disease who experienced a pregnancy. All patients were in complete or partial remission before conception with well-preserved renal function. Disease relapse and preeclampsia occurred in only 6.9 and 6.7% respectively and preterm births occurred in 23%. Overall adverse events were low, highlighting the importance of ensuring disease quiescence prior to conception.

In this Research Topic of *Frontiers in Medicine,* important associations between the clinical picture, histopathology and prognosis have been reported regarding frequent glomerular diseases. Identification of prognostic indicators among clinical, histopathological, or serological characteristics of patients is still critical in this field, in order to make decisions regarding planning of therapy at initial diagnosis and in the long-term. Until basic science finally move therapy of these important renal diseases from the exclusive reliance on glucocorticoids and toxic generalized immunosuppression to a new era of steroid-free, targeted, personalized renal care with agents that are safer, and more effective than the medications which have been the mainstays of therapy for a long time (28).

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Publisher's note

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Arterial-Arteriolar Sclerosis Is Independently Associated With Poor Renal Outcome in IgA Nephropathy Patients

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Aim: This study aimed to investigate the clinicopathological features and prognosis of immunoglobulin A nephropathy (IgAN) with arterial-arteriolar sclerosis (AS).

Methods: Patients with biopsy-proven IgAN from the West China Hospital of Sichuan University were retrospectively enrolled. Clinicopathological features were collected. Patients were categorized based on the presence and the severity of the AS. All the patients were regularly followed-up until a composite end point. The correlation between AS and prognosis of IgAN was assessed.

Results: A total of 1,424 patients were recruited and followed for 60.0 ± 28.7 months. Patients with AS tended to have older age, higher blood pressure, heavier proteinuria, higher serum creatinine, uric acid, and total triglyceride (TG). Meanwhile, they were more likely to have a lower estimated glomerular filtration rate (eGFR), hemoglobin, and albumin. At the end of follow-up, 126 patients in the AS group and 47 patients in the non-AS group had reached the composite end point ($p < 0.001$). AS was associated with the renal outcome (log-rank $p < 0.001$) and was an independent risk factor for the progression of IgAN ($p = 0.049$). The severity of AS was associated with renal outcomes (log-rank $p < 0.001$) and there was a trend that it might serve as an independent risk marker for progression of IgAN. In the subgroup analysis, patients presenting with AS and lower eGFR, albumin, and hemoglobin or higher proteinuria, uric acid, and TG had a significant trend for a shorter time to reach the end point (log-rank $p < 0.001$).

Conclusion: AS was commonly seen in patients with IgAN and was independently associated with the poor prognosis.

Keywords: IgA nephropathy, end-stage renal disease, renal survival, chronic kidney disease, arterial-arteriolar sclerosis

INTRODUCTION

Immunoglobulin A nephropathy (IgAN) is the most common primary chronic glomerular disease globally (1). It is characterized by the predominance of IgA deposits in the mesangium and requires renal biopsy for diagnosis (2). The pathological features of IgAN on light microscopy vary significantly among patients and can be reflected in the variable clinical features (3). The Oxford classification of IgAN has identified five widely validated factors: mesangial hypercellularity (M), endocapillary hypercellularity (E), segmental sclerosis (S), interstitial fibrosis/tubular atrophy (T), and crescents (C) to have independent value in predicting renal outcomes (4, 5). Besides these parameters, intrarenal arterial and arteriolar lesions, including thickening of the intimal wall and hyaline, can also be observed commonly in 40–55% of patients with IgAN (6–9). Intrarenal arteries and arterioles are responsible for supply of oxygen and nutrients, the lesions of which may lead to chronic hypoxia and renal injury (10). However, its effect on the progression of IgAN is still controversial and its relationship with other clinicopathological factors remains unclear. This retrospective study mainly focuses on arterial-arteriolar sclerosis (AS) and its relation with the renal outcome and other identified risk factors for poor kidney prognosis.

METHODS

Participants

From February 2009 to September 2017, a total of 1,545 renal biopsy-proven patients with IgAN from the West China Hospital of Sichuan University were evaluated for this retrospective study. Diagnosis of IgAN was based on the Kidney Disease: Improving Global Outcomes (KDIGO) Clinical Practice Guidelines for Glomerulonephritis (11). Patients with systemic lupus erythematosus (SLE), Henoch–Schönlein purpura (HSP), diabetes, liver disease, or malignancy were not included in this study. After 30 patients with incomplete data, 14 patients with a follow-up $<$ 1 year and 77 patients with fewer than 8 glomeruli in the renal biopsy slides were excluded; the remaining 1,424 patients were included in this study. This study was conducted following the Declaration of Helsinki principles and was approved by the Medical Ethical Committee of West China Medical School, Sichuan University (FF-33-2019). Written informed consent was obtained from all the patients.

Renal Histopathology

Renal biopsies were examined by immunofluorescence microscopy, light microscopy, and electron microscopy. H&E stain, Masson's trichrome stain, periodic acid-Schiff (PAS) stain, and periodic acid silver methenamine (PASM) stain were performed to assess the pathological features and AS. The lesions were reviewed by an experienced pathologist and a nephrologist according to the Oxford classification of IgAN (MEST-C scores): mesangial score $\leq$ 0.5 (M0) or $>$0.5 (M1); segmental glomerulosclerosis absent (S0) or present (S1); endocapillary hypercellularity absent (E0) or present (E1); tubular atrophy/interstitial fibrosis $<$ 25% (T0), 26–50% (T1), or $>$50% (T2); and crescents absent (C0), $<$25% (C1), $\geq$25% (C2). AS was defined as the presence of arterial intimal thickening or arterial-arteriolar hyaline changes in efferent arterioles, afferent arterioles, artery, and arterioles in the interstitium. Furthermore, intimal thickening was scored 0–2 according to the Oxford classification (12): score 0, no intimal thickening is present; score 1, intimal thickening less than the thickness of the media; and score 2, intimal thickening more than the thickness of the media. Hyaline change was assessed as presence or absence. Microangiopathy (MA) was defined as the presence of thrombi, endothelial swelling or denudation, intramural fibrin, or intimal swelling in the small artery and/or arterioles.

Clinical Data Collections

The baseline was established at the time of kidney biopsy. We systematically recorded sex, age, systolic blood pressure, diastolic blood pressure, and laboratory data including serum creatinine (SCr), estimated glomerular filtration rate (eGFR), hemoglobin (Hb), serum uric acid (UA), serum albumin (ALB), total serum cholesterol (TC), serum triglyceride (TG), 24-h proteinuria, and hematuria. eGFR was calculated according to the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula. Anemia was defined as Hb $<$ 120 g/L in male or Hb $<$ 110 g/L in female. Hyperuricemia was defined as UA $>$ 420 μmol/L in males or UA $>$ 360 μmol/L in females. Persistent hematuria was defined as urinary red blood cell more than 5 per high power field (HPF).

Treatment

Medications during follow-up were also collected. All the patients received optimized supportive treatment with full-dose angiotensin-converting-enzyme inhibitor (ACEI) or angiotensin receptor blockers (ARBs) and blood pressure was controlled below 140/90 mm Hg. Corticosteroids therapy (0.5–1 mg/kg prednisone daily, tapering down within 6–8 months) were used in patients with persistent proteinuria (protein excretion $>$ 1 g/d) and receiving a maximum dose of ACEI/ARB. Immunosuppressant therapy (cyclophosphamide 2 mg/kg daily for 3 months or mycophenolate mofetil 1–2 g daily for 6–8 months) was used based on the pathological classification and clinical severity.

Outcomes

The primary end point was a composite end point defined as a $\geq$50% reduction in eGFR, end-stage renal disease (ESRD), or death. ESRD was defined as eGFR $\leq$ 15 mL/min/1.73 m^2 or the requirement for renal transplantation or maintenance dialysis.

Statistics Analysis

Normally distributed variables were presented as mean $\pm$ SD and compared by using the Student's *t*-test. Non-normally distributed variables were expressed as median with interquartile ranges and analyzed with the Mann–Whitney *U* test. Categorical variables were described as the frequency with percentage and compared with the chi-squared test and the Fisher's exact test. The Kaplan–Meier method was used to analyze the survival probability between AS groups and other subgroups and survival curves were

TABLE 1 | Characteristics of immunoglobulin A nephropathy (IgAN) patients with or without arterial-arteriolar sclerosis (AS).

	All (n = 1,424)	Non-AS (n = 750)	AS (n = 674)	*P*-value
Clinical features				
Age, y	34.4 ± 11.1	32.1 ± 10.5	36.9 ± 11.2	<0.001
Sex (male)	640 (44.9)	330 (44.0)	310 (46.0)	0.45
Hypertension, %	405 (28.4)	124 (16.5)	281 (41.7)	<0.001
Proteinuria > 1 g/d	970 (68.1)	452 (63.3)	518 (76.9)	<0.001
Persistent hematuria, %	1,166 (81.9)	638 (85.1)	528 (78.3)	0.001
SCr, μmol/L	84.0 (65.2–110.0)	75.0 (61.4–05.7)	96.0 (73.0–130.7)	<0.001
eGFR, mL/min/1.73 m^2	89.8 ± 33.1	101.3 ± 30.2	77.1 ± 31.5	<0.001
eGFR < 60 mL/min/1.73 m^2, %	304 (21.3)	84 (11.2)	220 (32.6)	<0.001
Anemia, %	195 (13.7)	75 (10.0)	120 (17.8)	<0.001
Uric acid, μmol/L	374.4 (301.0–442.0)	348.5 (285.2–418.7)	387.5 (316.0–460.25)	<0.001
Hyperuricemia, %	596 (41.9)	257 (34.3)	339 (50.4)	<0.001
Albumin, g/L	39.0 ± 6.51	39.4 ± 6.82	38.7 ± 6.13	0.046
TC, mmol/L	5.04 ± 1.52	5.02 ± 1.63	5.06 ± 1.40	0.59
TG, mmol/L	1.85 ± 1.33	1.72 ± 1.34	1.99 ± 1.31	<0.001
CKD stage, %				
Stage 1	751 (52.7)	504 (67.2)	247 (36.6)	<0.001
Stage 2	369 (25.9)	162 (21.6)	207 (30.7)	<0.001
Stage 3	248 (17.4)	76 (10.1)	172 (25.5)	<0.001
Stage 4	52 (3.7)	8 (1.1)	44 (6.5)	<0.001
Stage 5	4 (0.3)	0 (0)	4 (0.6)	0.35
Pathologic features, %				
M1	1,075 (75.5)	503 (67.1)	572 (84.9)	<0.001
E1	67 (4.7)	24 (3.2)	43 (6.4)	0.005
S1	861 (60.5)	401 (53.5)	460 (68.2)	<0.001
T1-T2	294 (20.6)	60 (8.0)	234 (34.7)	<0.001
C0/C1-C2	325 (22.8)	142 (18.9)	183 (27.2)	<0.001
Medications, %				
Supportive treatment	606 (42.6)	350 (46.7)	256 (38.0)	<0.001
Steroids only	527 (37.0)	264 (35.2)	263 (39.0)	0.138
Immunosuppression and/or steroids	291 (20.4)	136 (18.1)	155 (23.0)	<0.001
Follow-up				
Duration, mon	60.0 ± 28.7	62.4 ± 29.1	57.2 ± 28.1	0.001
Composite endpoint, %	173 (12.1)	47 (6.3)	126 (18.7)	<0.001

Values presented as number (percentage), mean ± SD, or median (interquartile range).
SCr, serum creatinine; eGFR, estimated glomerular filtration rate; TC, total cholesterol; TG, triglyceride; M1, mesangial hypercellularity; E1, endocapillary hypercellularity; S1, segmental glomerulosclerosis; T1–T2, tubular atrophy/interstitial fibrosis > 25%; C1-C2, crescents > 0.

compared with the log-rank test. The univariate and multivariate Cox proportional hazards regression analysis were performed to explore the influence of variables on the composite end point. The relationship between AS and other variables was assessed by the logistic regression. A two-tailed $p < 0.05$ was considered as statistically significant. All the statistical analysis was performed with the Statistical Package for the Social Sciences (SPSS) statistics version 26.0 software (IBM Corporation, Armonk, New York, USA).

RESULTS

Demographic and Clinical Characteristics

A total of 1,424 biopsy-proven patients with IgAN from the West China Hospital of Sichuan University were enrolled in this retrospective study. Demographic, clinical, and pathological characteristics of included patients are shown in **Table 1**. Patients were followed for 60.0 ± 28.7 months. All the patients were categorized according to the presence of AS. Among all the 1,424 patients, 674 patients (310 males and 364 females) presented AS, while 750 patients (330 males and 420 females) presented non-AS. Besides, 187 patients (12.7%) also had hyaline changes. The mean age of patients in the AS group was 36.9 ± 11.2 years and the mean age of patients in the non-AS group was 32.1 ± 10.5 years. Compared with the non-AS group, patients presenting with the AS group had more tendency to have higher systolic blood pressure (SBP) and diastolic blood pressure (DBP) (SBP: 132.1 ± 19.3 vs. 123.4 ± 16.0, $p < 0.001$; DBP: 86.6 ± 14.4 vs. 80.1 ± 11.5, $p < 0.001$) and hypertension (281 vs. 124,

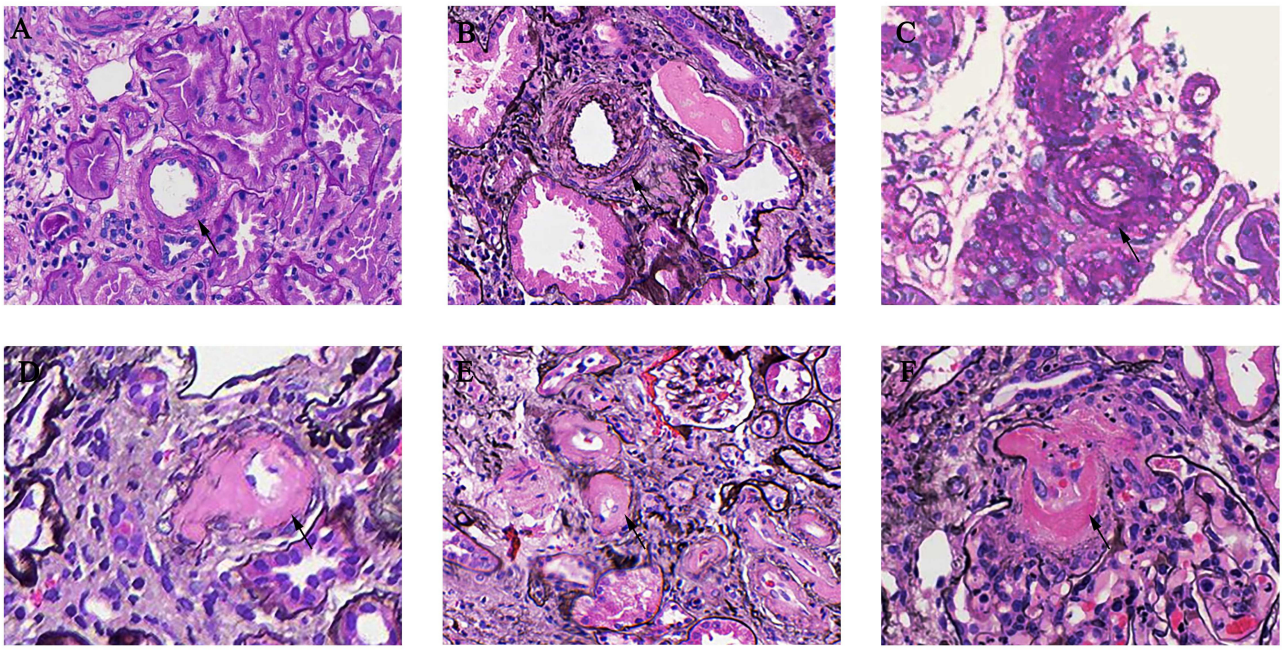

FIGURE 1 | Arterial-arteriolar lesions in immunoglobulin A nephropathy (IgAN). **(A)** Normal artery [periodic acid-Schiff (PAS) stain]. **(B)** Arteries with intimal thickening less than the thickness of the media [periodic acid silver methenamine (PASM) stain]. **(C)** Arteries with intimal thickening more than the thickness of the media (PASM stain). **(D)** Arteries with hyaline changes (PAS stain). **(E)** Arteries with hyaline changes (PASM stain). **(F)** Microangiopathic lesions (PASM stain).

$p < 0.001$). Laboratory findings showed that patients with AS had more proteinuria [1.86 (1.00–3.00) vs. 1.09 (0.64–2.35), $p < 0.001$], higher serum creatinine [96.0 (73.0–130.7) vs. 75.0 (61.4–50.7), $p < 0.001$], serum uric acid [387.5 (316.0–460.25) vs. 348.5 (285.2–418.7), $p < 0.001$], and total TG (1.99 ± 1.31 vs. 1.72 ± 1.34, $p < 0.001$); however, they had lower eGFR (77.1 ± 31.5 vs. 101.3 ± 30.2, $p < 0.001$), Hb (130.8 ± 21.0 vs. 134.6 ± 18.8, $p < 0.001$), and albumin (38.7 ± 6.13 vs. 39.4 ± 6.82, $p = 0.046$). With respect to treatment, a large proportion of patients with AS received immunosuppression and/or steroids (23.0 vs. 18.1%, $p < 0.001$) and they were less likely to be treated with supportive treatment (38.0 vs. 46.7%, $p < 0.001$) only.

Histopathological Characteristics

As shown in **Table 1**, mesangial hypercellularity was found in 572 (84.9%) patients in the AS group compared with 503 (67.1%) patients with the non-AS group ($p < 0.001$). There was also a significant trend of greater prevalence of endocapillary hypercellularity (6.4 vs. 3.2%, $p = 0.005$), segmental glomerulosclerosis (68.2 vs. 53.5%, $p < 0.001$), tubular atrophy/interstitial fibrosis (34.7 vs. 8.0%, $p < 0.001$), and crescents (27.2 vs. 18.9%, $p < 0.001$) among patients with AS compared to patients with non-AS. In our cohort, 74 patients (5.20%) presented with MA. Endothelial swelling was observed in 71 patients (4.99%) and 5 patients (0.35%) with thrombi. Different arterial-arteriolar lesions were shown in **Figure 1**.

Outcomes

At the end of the follow-up, a total of 173 patients (12.1%) had reached the composite end point. As shown in **Table 1**, there were 126 patients (18.7%) in the AS group progressed to composite end point compared with 47 patients (6.3%) in the non-AS group ($p < 0.001$).

The Kaplan–Meier analysis revealed that AS was significantly associated with poor renal outcome and the survival rate of patients in the AS group was much lower than that of patients in the non-AS group (log-rank $p < 0.001$, **Figure 2A**). The severity of AS was associated with renal outcomes. Patients with intimal thickening less than the thickness of the media (score 1) had a lower survival rate than patients without AS (score 0), meanwhile patients with intimal thickening more than the thickness of the media (score 2) had the worst renal outcome among three groups (log-rank $p < 0.001$, **Figure 2B**). However, there was no difference in composite end point between the hyaline changes groups (log-rank $p = 0.91$) and the MA groups (log-rank $p = 0.110$).

The subgroup analysis categorized based on eGFR < 60 ml/min/1.73 m^2, proteinuria > 1 g/d, albumin < 30 g/l, anemia, uric acid, and TG suggested that patients both presenting with AS and eGFR < 60 ml/min/1.73 m^2 had a significant trend for a shorter time to reach end point than other subgroups. Similar results were found in proteinuria > 1 g/d ($p < 0.001$), albumin < 30 g/l ($p < 0.001$), anemia ($p < 0.001$), uric acid ($p < 0.001$), and TG ($p < 0.001$) subgroups (**Figure 3**).

The Cox proportional hazards regression analysis was performed to explore the risk factor of the progression of

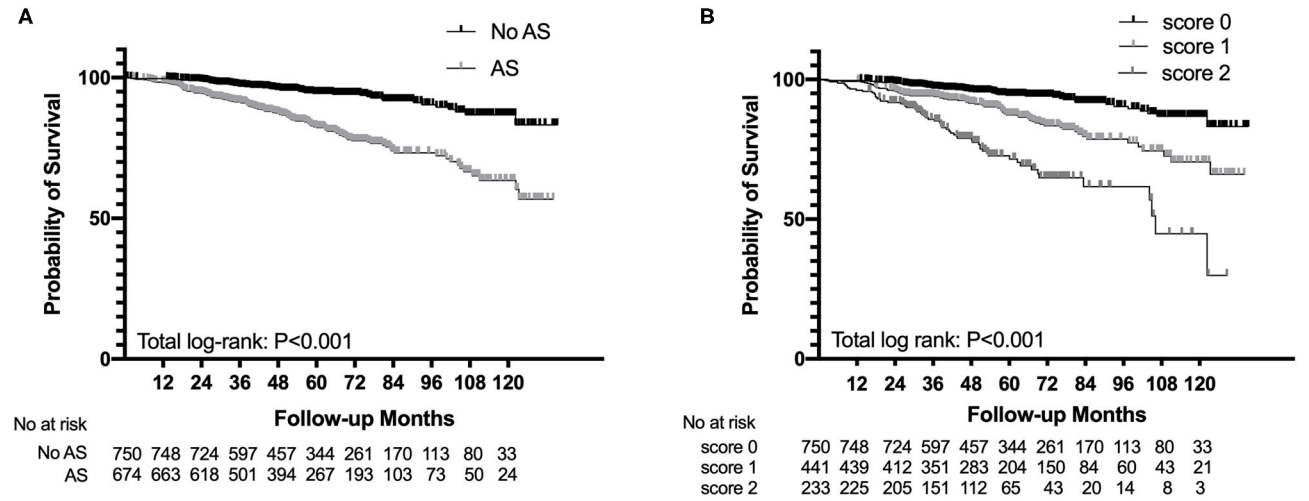

FIGURE 2 | Survival probability from end-stage renal disease (ESRD), a 50% reduction of estimated glomerular filtration rate (eGFR), or death in patients with IgAN with the presence of arterial-arteriolar sclerosis (AS) **(A)** and the severity of intimal thickening **(B)**.

IgAN. The univariate analysis showed that AS was significantly associated with a higher risk of the incidence of the composite end point [hazards ratio (HR) = 3.327, 95% CI: 2.379–4.653, $p < 0.001$]. As shown in **Table 2**, three models were used for the multivariate Cox proportional hazards regression analysis. After adjusting for age, gender, the Oxford classification of IgAN (MEST-C scores), MA, eGFR, proteinuria, hematuria, hyperuricemia, anemia, TG, ALB, and treatments (model 3), AS was an independent risk factor of the progression of IgAN to the composite end point (HR = 1.451, 95 CI%: 1.002–2.103, $p = 0.049$). In addition, age, segmental glomerulosclerosis, tubular atrophy/interstitial fibrosis, proteinuria, eGFR, hyperuricemia, albumin, and treatment were also significantly related to the development of composite end point. Besides, there seemed to be a trend that the severity of AS might serve as an independent risk marker for progression of IgAN, though the difference was not statistically significant in model 3 of the Cox proportional hazards regression analysis (score 0 vs. score 1: $p = 0.052$, score 0 vs. score 2: $p = 0.135$). These results may indicate that single-grade classification (presence and absence) may be a better indicator for renal outcomes than semi-quantification methods (score 0–2).

Relationship Between AS and Clinicopathological Features

As shown in **Table 3**, the logistic regression analysis was conducted to analyze the relationship between AS and clinicopathological features. It revealed that the existence of mesangial hypercellularity [odds ratio (OR) = 2.143, 95% CI: 1.596–2.879, $p < 0.001$], tubular atrophy/interstitial fibrosis (OR = 3.76, 95% CI: 2.623–5.389, $p < 0.001$), and crescents (OR = 1.433, 95% CI: 1.072–1.915, $p = 0.015$) were significantly related to the presence of AS. Notably, proteinuria more than 1 g per 24 h (OR = 1.477, 95% CI: 1.123–1.943, $p = 0.005$), older than 30 years old (OR = 1.844, 95% CI: 1.427–2.383, $p < 0.001$), and hypertension (OR = 2.275, 95% CI: 1.718–3.014, $p < 0.001$) were also strongly suggested a tendency of the presence of AS.

DISCUSSION

In this retrospective study, AS was found in 43.7% of the whole cohort, similar to the Oxford study (4) and a Chinese cohort established by the Peking University (6). Moreover, we also found that AS had a significant influence on the progression of IgAN. The probability of a ≥50% reduction in eGFR, ESRD, or death during the follow-up was significantly higher in the AS group than patients with the non-AS group. The presence of AS was still an independent factor for the progression of the disease after adjusting for both the clinical and pathological variables, meanwhile the severity of AS did not show the same results, which indicated that the single-grade classification of AS might be a better indicator of the prognosis of IgAN than semi-quantification.

However, the effect of AS on the progression of IgAN was controversial in the previous studies. Russo et al. reported that patients with arterial diseases were at a higher risk of progression to death or ESRD compared to patients without it and patients having both the arterial disease and high uric acid showed a shorter time progressing to end point compared to those having only one risk factor, which was consistent with this study (7). Moreover, as Zhang et al. reported in a retrospective study, they also found that the presence of vascular lesions was associated with poorer renal outcomes (9). However, in the original Oxford study, arterial and arteriolar lesions were evaluated as artery score, which showed that arterial and arteriolar lesions were not associated with the rate

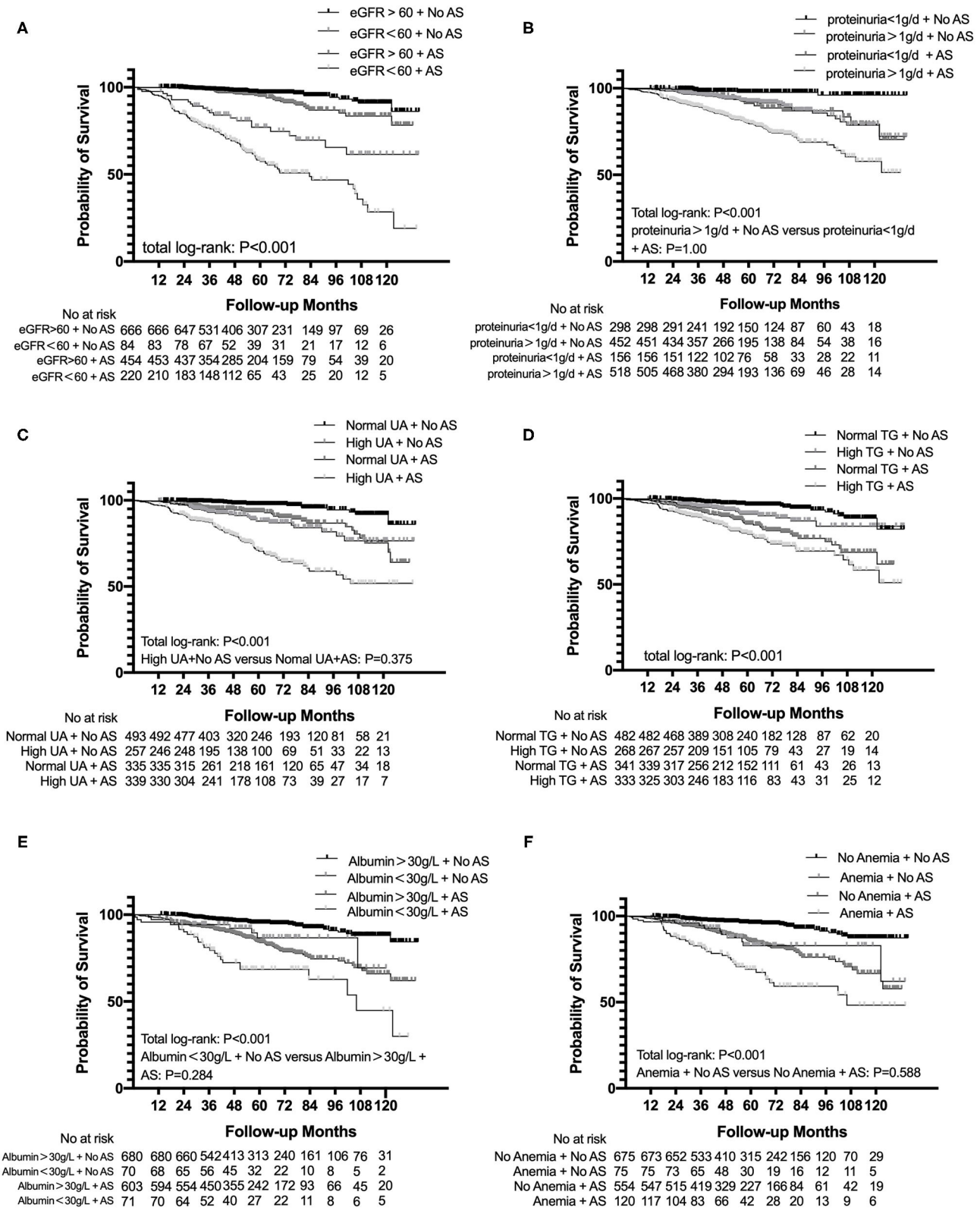

FIGURE 3 | Subgroup analysis for survival probability from ESRD, a 50% reduction of eGFR, or death in patients with IgAN with AS and eGFR **(A)**, proteinuria **(B)**, uric acid **(C)**, total triglyceride **(D)**, albumin **(E)**, and hemoglobin **(F)** levels. eGFR, estimated glomerular filtration rate; UA, uric acid; TG, triglyceride.

TABLE 2 | The Cox proportional hazards regression models for composite endpoint in patients with IgAN.

Variable	Univariant		Model 1		Model 2		Model 3	
	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*	HR (95% CI)	*P*
AS	3,327 (2.379–4.653)	<0.001	1.738 (1.206–2.505)	0.003	1.986 (1.396–2.826)	<0.001	1.451 (1.002–2.103)	0.049

Model 1 adjusted age, gender, Oxford classification of IgA (MEST-C scores), and microangiopathy.
Model 2 adjusted age, gender, eGFR, proteinuria, hematuria, hyperuricemia, anemia, total TG, albumin, and treatments (supportive treatment, steroids only, and immunosuppression and/or steroids).
Model 3 adjusted covariates in model 1 and model 2.

TABLE 3 | The logistic regression models for the presence of AS.

Variable	OR	95% CI	*P*-value
M	2.143	1.596–2.879	<0.001
E	1.564	0.876–2.794	0.131
S	1.237	0.961–1.592	0.099
T0/T1-T2	3.76	2.623–5.389	<0.001
C0/C1-C2	1.433	1.072–1.915	0.015
Proteinuria > 1 g/d	1.477	1.123–1.943	0.005
eGFR < 60 mL/min/1.73 m^2	1.326	0.934–1.881	0.114
Hyperuricemia	0.98	0.755–1.273	0.88
Hyperlipemia	1.277	0.990–1.648	0.06
Hypercholesteremia	0.77	0.587–1.011	0.059
Anemia	1.08	0.746–1.566	0.682
Hypoalbuminemia	1.111	0.716–1.724	0.638
>30 years old	1.844	1.427–2.383	<0.001
Hypertension	2.275	1.718–3.014	<0.001

OR, odds ratio; eGFR, estimated glomerular filtration rate; M1, mesangial hypercellularity; E1, endocapillary hypercellularity; S1, segmental glomerulosclerosis; T1–T2, tubular atrophy/interstitial fibrosis > 25%; C1-C2, crescents > 0.

of renal function decline. A similar conclusion also has been drawn by Cai et al. (6). Artery score has not been discussed in the further Oxford study (5). The possible causes for this inconsistency may arise from the several aspects. In this study, most of our patients were resident in the Southwest of China and the other cohort mentioned above mainly originated from Caucasian or Northern China, different populations among different cohorts were considered as one important reason. Also, the baseline characteristics were different among cohorts. There were more patients with severe pathological classifications and heavier proteinuria in our cohorts, which can result in the different treatment methods proportion between the studies. Furthermore, renal AS is considered as a chronic lesion and its effects on renal survival also may not reflect thoroughly in a short time of follow-up. Therefore, long-time follow-up was needed to explore the relationship between AS and renal outcomes. This study had a comparatively larger sample size and a longer follow-up duration than other studies; therefore, the duration of follow-up may also partly explain the inconsistent conclusions.

In our center, 74 patients (5.20%) presented with MA. Endothelial swelling was observed in 71 patients (4.99%) and only 5 patients (0.35%) had thrombi. However, there was no difference in composite end point between the MA groups (log-rank $p = 0.110$). This may be due to the low prevalence of MA in our cohort.

In this study, patients with AS tended to be older and have higher blood pressure, heavier proteinuria, a higher level of serum creatinine, serum uric acid, and total TG. Meanwhile, they were more likely to have a lower eGFR, Hb, and albumin. Also, in the logistic regression analysis, the existence of mesangial hypercellularity, tubular atrophy/interstitial fibrosis, crescents, persistent proteinuria, older age, and uncontrolled hypertension before diagnosis were also strongly suggested a higher tendency of the presence of AS. These results were similar to recently published studies (6–9). Among all these variables, most of the variables were reported to be risk factors of the progression of the disease including mesangial hypercellularity, tubular atrophy/interstitial fibrosis, crescents, eGFR, persistent proteinuria, anemia, total TG, and high level of uric acid (13–17). This was also confirmed in the subgroup analysis that eGFR, persistent proteinuria, high uric acid, TG, reduced Hb, and albumin have a synergic effect with AS on renal outcomes. It is well-known that hypertension and total TG were closely related to the pathogenesis of arterial sclerosis (18, 19). However, AS also has been observed in patients without hypertension. In this study, we confirmed that 58.3% of AS did not have hypertension. This suggested that hypertension may not be the only reason that leads to AS. Moreover, in a recently published study, a high level of uric acid seemed to have a synergic effect with the arterial lesions on the renal outcome, caused by the proliferation of vascular smooth muscle cell activated by uric acid through mitogen-activated protein kinases and stimulating cyclooxygenase-2 and platelet-derived growth factor (GF) synthesis (7, 20). With respect to eGFR and persistent proteinuria, these factors were already identified to be strong prediction factor of the poor renal outcome for patients with IgAN in many former large-scale studies, which have been already applied as instructions for treatment in the KDIGO guidelines (11). Serum albumin is closely related to urinary protein excretion and also it was reported as an independent risk factor of prognosis of IgAN (21). Anemia and AS may also have a synergistic effect on chronic renal hypoxia and contribute to the progression of the disease (22).

To evaluate the effect of different variables on the progression of the disease, the multivariate Cox proportional hazards regression analysis was performed and both the age, gender, clinical variables, pathological variables, and medications were

all taken into account. Notably, all the hypertension cases in our cohort were carefully treated with antihypertensive therapy and the blood pressure reached targeted blood pressure (within 140/90 mm Hg) during the follow-up. The multivariate Cox proportional hazards regression analysis confirmed that AS was an independent risk factor for renal progression of patients with IgAN.

The possible mechanism for AS contributing to the progression of the disease may be hypoxia. It was reported that arteriolar hyalinosis was a possible surrogate marker of reduced interstitial blood flow and hypoxia in glomerulonephritis (23). AS leads to the impairment of arterial and arteriolar functions on offering oxygen and nutrients to tubular and interstitial cells, resulting in chronic ischemia of the tubular cells and interstitium, together with the distortion and loss of peritubular capillaries (24). Hypoxia is a profibrogenic factor for tubular cell and accelerates interstitial fibrosis, which can largely reduce oxygen diffusion efficiency, and then forms a vicious circle (25). Hypoxia can also lead to podocyte injury by overexpressing hypoxia inducible factor-1 (HIF1). Podocytes can present with podocyte epithelial-mesenchymal transition (EMT), slit-diaphragm dysfunction, foot process effacement, and cytoskeletal derangement due to accumulation of HIF1, which contributed to pathology of glomerular diseases and proteinuria (26).

This study has some limitations that deserve to be mentioned. Firstly, patients recruited in this study were mainly from the Southwest China and this study is a retrospective single-center study. The baseline between the two groups was not entirely balanced, which was a common limitation of this retrospective study. We performed multivariate analysis to eliminate the confounding factors, but this study may still not suitable for all the regions of the world. Secondly, the follow-up duration was relatively short compared to the progressive speed of IgAN. Thus, the findings still need further confirmation.

CONCLUSION

Arterial-arteriolar sclerosis was commonly seen in patients with IgAN and was independently associated with the poor prognosis.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Medical Ethical Committee of West China Medical School, Sichuan University. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

LD: design of the work, analysis and interpretation of data, and drafting and revising the manuscript. JT and SW: analysis and interpretation of data. FL: acquisition and analysis of data. AQ and ZJ: analysis of data. ZZ and XZ: acquisition of data. YT: conception and supervision of the work. WQ: conception and design of the work. All authors involved in the final approval of the manuscript to be published.

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Clinicopathologic Implications of Complement Genetic Variants in Kidney Transplantation

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Genetic testing has uncovered rare variants in complement proteins associated with thrombotic microangiopathy (TMA) and C3 glomerulopathy (C3G). Approximately 50% are classified as variants of uncertain significance (VUS). Clinical risk assessment of patients carrying a VUS remains challenging primarily due to a lack of functional information, especially in the context of multiple confounding factors in the setting of kidney transplantation. Our objective was to evaluate the clinicopathologic significance of genetic variants in TMA and C3G in a kidney transplant cohort. We used whole exome next-generation sequencing to analyze complement genes in 76 patients, comprising 60 patients with a TMA and 16 with C3G. Ten variants in complement factor H (*CFH*) were identified; of these, four were known to be pathogenic, one was likely benign and five were classified as a VUS (I372V, I453L, G918E, T956M, L1207I). Each VUS was subjected to a structural analysis and was recombinantly produced; if expressed, its function was then characterized relative to the wild-type (WT) protein. Our data indicate that I372V, I453L, and G918E were deleterious while T956M and L1207I demonstrated normal functional activity. Four common polymorphisms in *CFH* (E936D, N1050Y, I1059T, Q1143E) were also characterized. We also assessed a family with a pathogenic variant in membrane cofactor protein (*MCP*) in addition to *CFH* with a unique clinical presentation featuring valvular dysfunction. Our analyses helped to determine disease etiology and defined the recurrence risk after kidney transplant, thereby facilitating clinical decision making for our patients. This work further illustrates the limitations of the prediction models and highlights the importance of conducting functional analysis of genetic variants particularly in a complex clinicopathologic scenario such as kidney transplantation.

Keywords: kidney transplantation, complement, complement regulators, atypical hemolytic uremic syndrome, variants of uncertain significance, thrombotic microangiopathy, C3 glomerulopathy

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INTRODUCTION

Two prototypical complement-mediated kidney diseases are atypical hemolytic uremic syndrome (aHUS) and C3 glomerulopathy (C3G) (1). Atypical hemolytic uremic syndrome is a primary complement-mediated thrombotic microangiopathy (TMA) characterized by hemolytic anemia, thrombocytopenia, and acute kidney injury (AKI) (2). C3 glomerulopathy is a group of nephritides in which complement activation leads to a predominant C3 fragment glomerular deposition as seen by immunofluorescence (3). It is subclassified as dense deposit disease or C3 glomerulonephritis (C3GN) based on the type and location of deposits as visualized by electron microscopy.

Recent progress in our understanding of the role of complement dysregulation and efficacy of complement blockade has revolutionized the clinical course and outcome of these diseases (4). Therapeutic advances render kidney transplantation as a viable option for patients with aHUS and C3G who previously were considered non-transplantable.

However, many challenges remain. First, aHUS may be mimicked by other disease processes, currently classified under secondary TMAs, which include infections, pregnancy, autoimmune conditions, and graft rejection (3). Kidney transplantation poses an especially problematic setting since there are multiple potential triggers (transplant surgery, drugs, rejection, and infections) for TMA development. Consequently, it can be "tricky" for transplant clinicians to distinguish aHUS from these secondary TMAs. However, the distinction is critical since complement-mediated TMAs are often non-responsive to conservative measures (removal of the offending drug or treating the underlying rejection/infection) (5). Second, both aHUS and C3G have a high risk of recurrence after kidney transplantation and the outcome can vary depending on the underlying complement abnormality (6, 7). Genetic variants in complement proteins are identified in approximately 60% of patients with aHUS, 10–60% of patients with secondary causes, and 30–40% of patients with C3G (8). However, <50% of the identified variants have a known functional consequence and are classified by the American College of Medical Genetics (ACMG) as variants of uncertain significance (VUS). Thus, the inability to ascertain the functional impact of a VUS poses another major barrier for clinical management after kidney transplantation. Finally, decisions about accepting living related donors for these patients can be complicated since there may be risk of recurrent disease in the recipient and *de novo* disease in the donor if the underlying etiology of disease is unclear.

We have previously published functional studies on VUS in complement factor I (*CFI*) in patients with a TMA that facilitated establishing their clinicopathologic relevance (9, 10). We have also characterized complement factor H (*CFH*) and *CFI* variants identified in patients with age-related macular degeneration (AMD), a leading cause of blindness in the developed world. These findings helped explain the early onset of advanced AMD in the patients as well as the high burden of disease in their families (10, 11). We now present studies on the functional characterization of rare genetic variants and single nucleotide polymorphisms (SNPs) in *CFH* in our kidney transplant cohort (**Figure 1**). This study highlights how a systematic and comprehensive analysis can help to address the challenges described above, fill the knowledge gap relative to underlying mechanism of disease, and facilitate therapeutic decisions in a kidney transplant population. Our work also emphasizes several additional key concepts relative to limitations of the computational predictive models, the role of multiple *CFH* variants (common and rare), the impact of concomitant variants in *CFI* and membrane cofactor protein (*MCP*, *CD46*), and modifications due to underlying diseases including especially lupus.

MATERIALS AND METHODS

Study Population

Patients diagnosed with a TMA or with biopsy-proven C3G between 2015 and 2020 at Washington University School of Medicine (WUSM) were evaluated. These patients were identified during the pretransplant evaluation at our center or were referred to us from other centers. Patients with a TMA included those with a biopsy-proven diagnosis in their native kidney or those who developed a *de novo* TMA after transplantation in the renal allograft. The diagnosis of C3G was based on the 2013 consensus report guidelines which required intensity of C3 staining at least two orders of magnitude greater than any other immunoreactant (i.e., IgG, IgM, IgA, and C1q) on a scale of 0–3 (including 0, trace, 1+, 2+, 3+) in native kidney or allograft (11). This research followed the tenets of the Declaration of Helsinki. The study was approved by the institutional review board at Washington University School of Medicine in St. Louis, MO. Informed consent was obtained from each subject.

Sequencing

Next generation sequencing was performed by the Genomic and Pathology Services (GPS) at WUSM (12). The clinically validated aHUS/C3G next generation sequencing-based assay is derived from the Agilent Clinical Research Exome capture reagent (Agilent Technologies, Inc., Santa Clara, CA), with analysis of all exonic sequences for 13 genes (*ADAMTS13, C3, CD46, CFB, CFH, CFHR1, CFHR2, CFHR3, CFHR4, CFHR5, CFI, DGKE, THBD, MMACHC*, and *PLG*). CFHR3-CFHR1 copy number was assessed using multiplex ligation-dependent probe amplification (MLPA). All variants are reported according to Human Genome Variation Society (HGVS) nomenclature and classified based on the guidelines established by the joint consensus of the ACMG and the Association of Molecular Pathology (AMP). Based on these standards, variants are classified as: Level 1: pathogenic; Level 2: likely pathogenic; Level 3: variants of uncertain clinical significance (VUS); Level 4: likely benign; and Level 5: benign. The *CFH* reference wild-type (WT) sequence NM_000186.4 was employed for variant calling.

Molecular Engineering of Factor H CCP 1–8 and CCP 15–20 Fragments

Preparation of the FH CCP 18–20 vector has been described (14). A two-step DNA synthesis method was used to construct

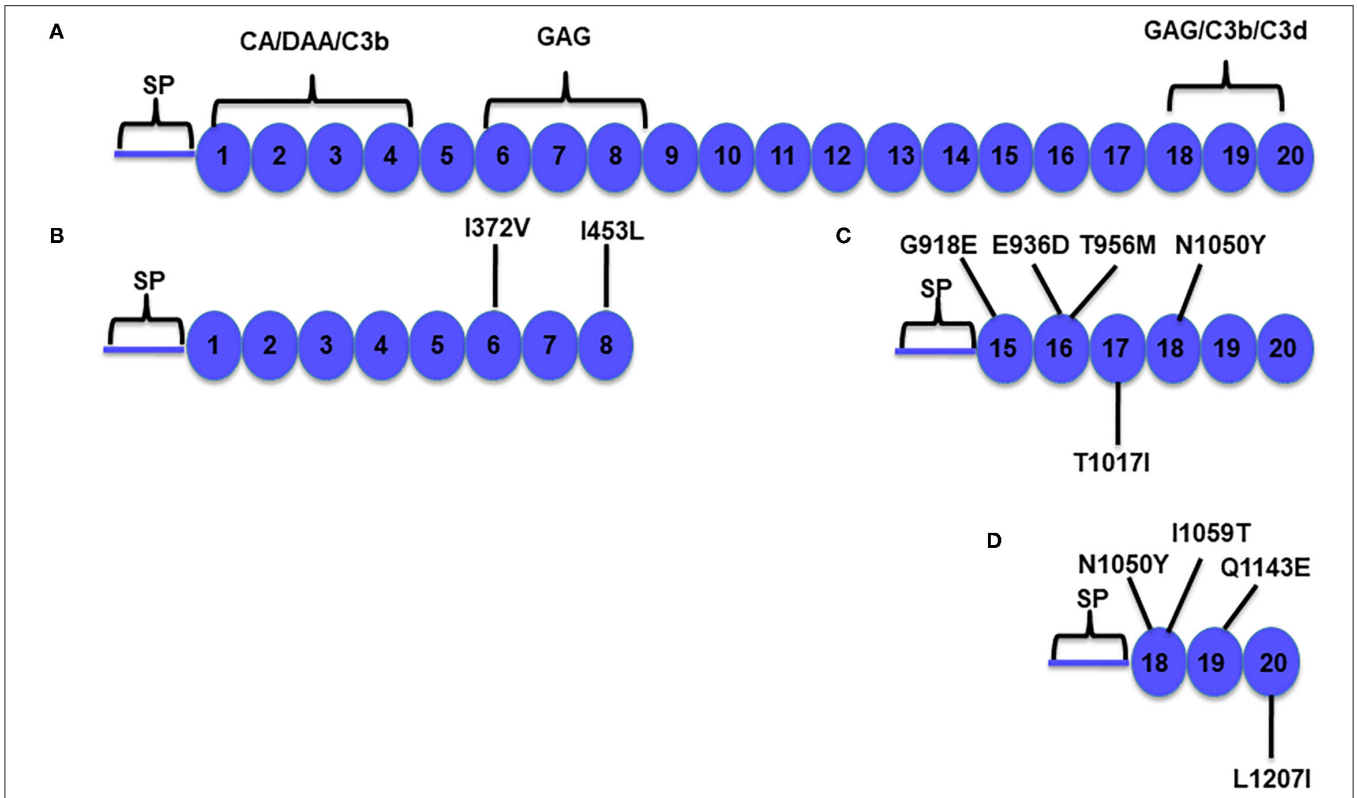

FIGURE 1 | Schematic diagram of Factor H (FH) and its functional domains. **(A)** Factor H is a 155-kDa plasma complement regulatory protein composed of 20 repeating homologous domains of approximately 60 amino acids each, known as complement control protein (CCP) repeats, short consensus repeats (SCRs), or sushi domains (12). Factor H prevents the formation and accelerates the decay of the alternative pathway C3 convertase (C3bBb; decay accelerating activity) and is a cofactor for Factor I-mediated cleavage and inactivation of C3b (cofactor activity). Factor H has fluid-phase and cell-surface regulatory capabilities (13). The N-terminal portion contains the regulatory domain that mediates the cofactor activity and decay accelerating activity (repeats 1–4). The surface binding recognition motifs are located in repeats 6–8 and 18–20. **(B–D)** Factor H constructs used to prepare the variants (as indicated). Residue numbering includes the 18 amino acids of the signal peptide (SP). CA, cofactor activity; DAA, decay accelerating activity; C3b, C3b binding site; C3d, C3d binding site; GAG, glycosaminoglycan binding site.

the FH CCP 1–8 and CCP 15–20 vectors (15). The CCP 1–8 and CCP 15–20 fragments were generated by PCR with a C-terminal 6× His tag. These products were used as templates for a second PCR reaction in which the outer primer contained an N-terminal signal peptide and an EcoRI restriction site. The PCR products were subsequently digested with EcoRI and BamHI and then ligated with the pSG5 vector. The fidelity of FH CCP 1–8, CCP 15–20, and CCP 18–20 pSG5 vectors was verified by DNA sequencing. Primers used for PCR amplification are listed in **Supplementary Table 1**.

Preparation and Expression of FH Variants

The FH variants (**Figures 1B–D**) were produced using the QuikChange XL site-directed mutagenesis kit (Agilent Technologies, Santa Clara, CA). Each *CFH* cDNA clone was sequenced. The variants were transfected into human embryonic kidney 293T cells and DMEM was replaced with serum-free OptiMEM® (Invitrogen, NY). Transient transfections were performed using the Xfect reagent (Takara Bio USA, Mountain View, CA). Supernatants were collected after 72 h, concentrated 80×, and stored in aliquots at −80°C (9).

Quantifying FH Variants by ELISA and Western Blotting

The quantity of each recombinant FH variant protein was determined by ELISA (9, 16). The microtiter plates were coated with capture antibodies (Abs) (A254 for CCP 1–8; A229 for CCP 15–20, and CCP 18–20, Quidel) at 2 μg/ml overnight at 4°C and blocked for 1 h at 37°C (in 10 mM Tris, 150 mM NaCl pH 7.4, 1% BSA, and 0.05% Tween 20). The WT, variant fragments and purified human FH (Complement Technologies, TX) were diluted, incubated for 1 h at 37°C, and then washed with PBS pH 7.4 containing 0.05% Tween 20 (PBS-T). A polyclonal goat antiserum to human FH (A312, San Diego, CA) was employed to detect bound FH using a standard ELISA protocol (9, 16). For Western blots, supernatants were electrophoresed under reducing conditions using 4–20% SDS-PAGE, transferred to nitrocellulose, and then probed with 1:5,000 mouse Monoclonal TetraHis Ab (Qiagen, USA), followed by a 1:10,000 HRP goat anti-mouse IgG (Abcam, Cambridge, MA) (**Figure 2**).

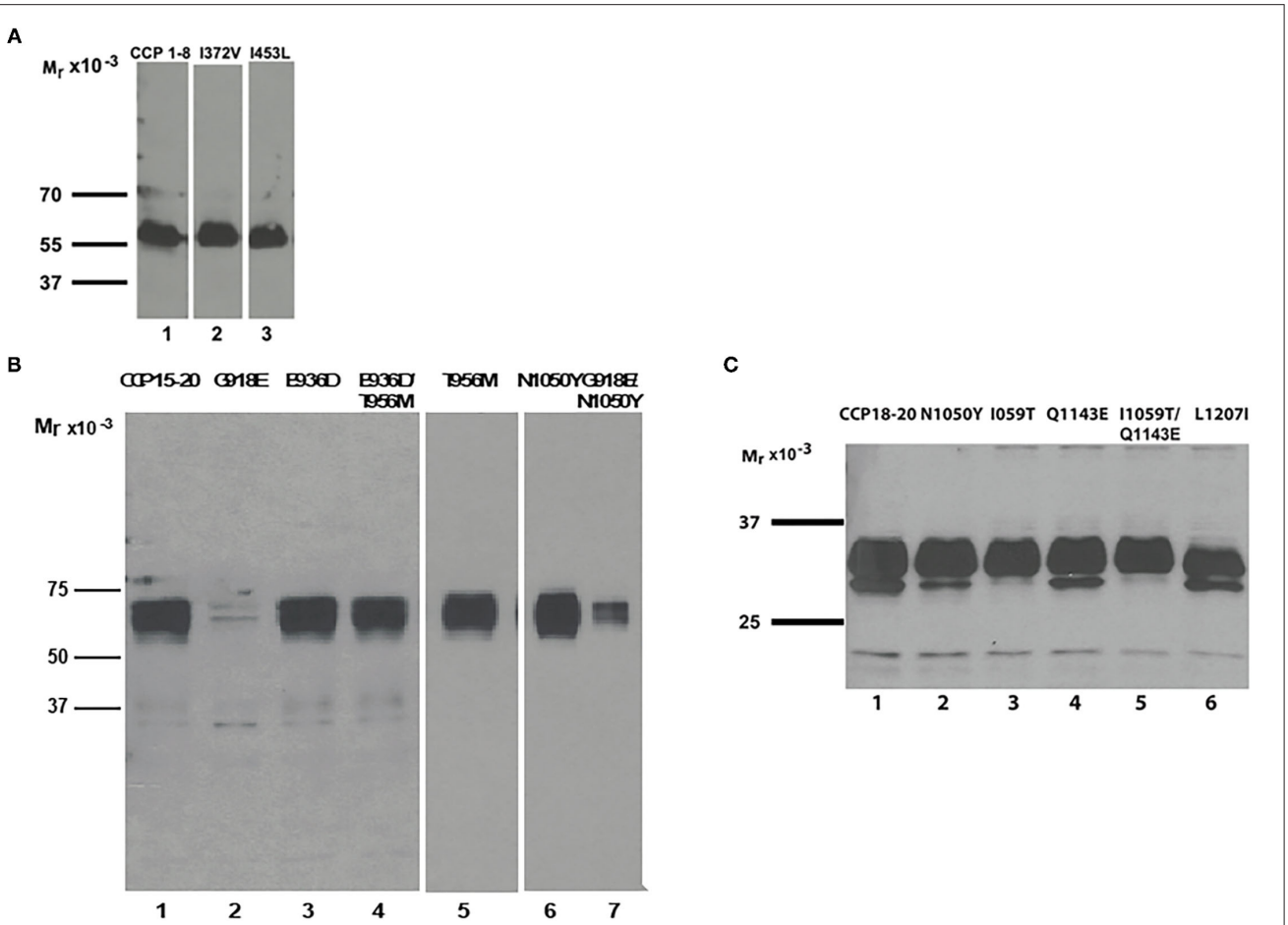

FIGURE 2 | Recombinant expression of CFH variants. Western blots of supernatants from transfected wild-type and variant constructs. **(A)** Recombinant expression of variants I372V (lane 2) and I453L (lane 3) shows secretion comparable to wild type CCP 1–8 (lane 1). **(B)** Wild type CCP 15–20 as control (lane 1). Secretion of variant protein G918E is undetectable (lane 2). The SNP N1050Y displays higher secretion than WT (lane 6). The combined variant G918E/N1050Y has markedly reduced secretion (lane 7). Variant T956M has normal secretion (lane 5). Combined variant T956M/E936D and the SNP E936D also demonstrate normal secretion (lanes 3 and 4). **(C)** Wild type CCP 18–20 as control (lane 1). The SNPs N1050Y, I1059T, Q1143E, and variant L1207I show normal secretion (lanes 2–6).

Functional Studies

The variants were produced as recombinant protein fragments of FH and their function was tested against the corresponding WT FH fragments and also the WT full-length protein. Patient serum was also used for fluid-phase and cell-surface assays. A genetic variant was considered to be deleterious if the protein was (a) not synthesized and/or not secreted *in vitro* (as measured by Western blot and ELISA) or (b) secreted in normal amounts but was dysfunctional based on any one of the assays outlined below.

Binding Assays

Variant and WT FH binding to C3b and heparin was assessed by ELISAs. Microtiter plates were coated with human C3b (37.5 nM for CCP 1–8; 15 nM for CCP 15–20 and CCP 18–20) or unfractionated heparin (2 mg/ml; Sigma-Aldrich) overnight at 4°C in $NaHCO_3$ buffer [50 mM for C3b binding (17); 100 mM for heparin binding, pH 9.6) (18)], followed by blocking with 4% BSA in PBS-T (15, 19). Samples were diluted in ELISA buffer and incubated for 1 h at 37°C. Bound FH was detected as described previously (9, 16). On at least three occasions, binding assays were performed employing serially diluted samples.

Cofactor Activity

Factor H proteins were diluted in physiologic salt buffer (10 mM Tris pH 7.4, 150 mM NaCl). C3b (15 ng) was incubated at 37°C with purified FH (200 ng), equivalent moles of WT CCP 1–8 or variant FH protein, and FI (20 ng) in a total reaction volume of 20 μl. Kinetic analyses were performed at 0, 5, 10, and 20 min. The samples were electrophoresed on a 4–20% gradient Tris-glycine gel and then transferred to nitrocellulose for Western blotting (9).

Serum-Based Assays

These included the sheep red blood cell hemolytic assay, the C3b deposition assay and the C3G biomarker panel. These tests were conducted at the Molecular Otolaryngology and Renal Research Laboratories (MORL), Iowa (https://morl.lab.uiowa.edu/). The hemolytic assay measures complement-mediated lysis of sheep erythrocytes secondary to activation of the alternative

pathway. The C3b deposition measures complement-mediated C3b deposition on the surface of cultured MES-13 cells as visualized using Alexa-488 labeled anti-C3 antibody.

Statistical Analysis

Statistical analyses were performed using Prism software version 8 (GraphPad). Comparisons between two groups were assessed using paired *t*-test (non-parametric). Comparisons among groups were performed using a one-way ANOVA with Dunnett's multiple comparison test ($P < 0.05$ was considered as significant).

RESULTS

A cohort of 76 kidney transplant patients (comprising 60 patients with a TMA and 16 with C3G) were identified and underwent genetic sequencing. Thirty-three of the 76 patients (∼ 44%) carried a variant in a complement protein (**Supplementary Table 2**). Twenty-one of the 33 variants (∼64%) were classified as a VUS. We have previously reported functional studies on VUS in *CFI* in this cohort that helped to establish their clinical significance (9). Variants in *CFH* were the focus of the current investigation. Ten rare genetic variants and four SNPs (E936D, N1050Y, I1059T, Q1143E) in *CFH* were identified (**Supplementary Tables 2**, **3**). Of the 10 *CFH* variants, four were classified as pathogenic (C611Stop, C853R, c.619 + 1G>A, c.3493 + 1G>A), five as VUS (I372V, I453L, G918E, T956M, and L1207I) and one as likely benign (T1017I). We investigated the effects of the five VUS in *CFH* in eight patients. We also functionally characterized the SNPs (alone and in combination with a rare variant). Two patients in one family also carried concomitant variants in *CFI* (K441R, classified as likely benign) and *MCP* (c.287-2A>G, classified as pathogenic).

We hereby present "real-life" cases of eight patients with a detailed clinical history for each. Comprehensive antigenic, functional, and structural analyses were conducted to determine the underlying etiology of disease which was key for clinical decision-making regarding treatment and risk of recurrence.

Complement-Mediated TMA Vs. Secondary TMA After Kidney Transplantation

Clinical History

Patient 1

A 45-year-old Asian female developed end-stage renal disease (ESRD) secondary to biopsy-proven lupus nephritis. She underwent a deceased donor kidney transplant (DDKT) in 2008, which was complicated on post-operative day 5 by a TMA in the allograft leading to a transplant nephrectomy (**Supplementary Figure 1**). The etiology of TMA was presumed to be the calcineurin inhibitor (CNI). Therefore, during the second DDKT in 2012, belatacept was used instead of a CNI. Two years later, the patient developed AKI requiring hemodialysis (HD). Allograft biopsy (not shown) revealed acute cellular rejection and endothelialitis which did not respond to conventional treatment with steroids and she remained dialysis dependent. Given the loss of two allografts due to TMA and pathologic evidence of endothelial cell injury, genetic testing for complement variants was performed during the evaluation for a third transplant (**Table 1**).

Patient 2

A 36-year-old African American female developed ESRD secondary to biopsy-proven lupus nephritis. She received a DDKT which failed within 1 year and she returned to dialysis. Allograft biopsy demonstrated arterioles with marked mucoid intimal edema and fragmented red cells. Laboratory results suggested a TMA (**Table 1**). Therefore, she underwent genetic testing during the evaluation for a second transplant.

For both patients described above, there was no family history of kidney disease. ADAMTS13 activity was normal and elevated levels of anti-phospholipid antibodies (APL Abs) or FH autoantibodies were not detected. Testing for Shiga toxin was also negative.

Genetic Analysis

We identified rare heterozygous *CFH* variants, I372V in patient 1 and I453L in patient 2 (**Table 1**). I372V has been reported in gnomAD with a MAF of 0.0008% in East Asians only and I453L with a MAF of 0.0032% in an individual of African ancestry. They were classified as VUS due to their rarity and lack of functional data or other evidence to support pathogenicity.

Structural Analysis

The I372 residue is a moderately conserved amino acid in CCP 6 (**Figure 3**) that is spatially adjacent to a putative GAG binding site in CCP 6–8 of the FH crystal structure (PDBID:2UWN and 2W80) (**Figures 4A,B**) (20, 21). The I372V substitution involves two hydrophobic amino acids. Both crystal structures (PDBID 2UWN and 2W80) demonstrate that the hydrophobic side chain of I372 is buried inside the CCP 6 protein core, away from the interacting surface. However, the alteration to a smaller hydrophobic residue V may influence the external surface of CCP 6 and its binding interactions through an internal structural rearrangement.

Sequence alignments of mammalian CCP 8 revealed that I453 is moderately conserved in 10 mammalian sequences (**Figure 3**) (22). Although I453 is spatially away from R444 (a putative heparin binding site) (19) (**Figure 4A**), the substitution of I to L may influence the external surface of CCP 8 and its binding interactions.

Antigenic and Functional Analyses

Factor H antigenic levels in both patients were in the normal range (**Table 1**). Secretion of the recombinantly produced variants (I372V and I453L) and their binding affinities to C3b were comparable to those of WT and full-length FH (**Figures 2**, **4C**; **Table 2**). Cofactor activity in our standard assay with the recombinant proteins was also normal (**Figure 4D**). However, the variants showed a modest increase in binding affinity for heparin compared to WT. Given the aggressive nature of the TMA, loss of multiple allografts, rarity of the variants, and the structural data supporting a possible deleterious effect, we conducted further investigation using serum-based assays. The samples were sent to MORL,

TABLE 1 | Genetic, immunologic, and clinical data from the TMA/C3G cohort at Washington University School of Medicine (WUSM).

Patients	Variants	MAF (%)		Allele count (gnomAD)/Number of homozygotes	C3 level (mg/dl)	C4 level (mg/dl)	Factor H (mg/dl)	FH autoantibody (≤22 unit/ml)	C3Nef (0.0–0.26)	Transplant history
		Total	Population							
1	I372V	0.0008	0.011 (East Asian)	2/0	72 (83–185)	19.2 (12–54)	66.2 (37–68)	Negative	Negative	2 failed
2	I453L*	0.0032	0.012 (African American)	1/0	57 (90–180)	11 (10–40)	38.8 (37–68)	Negative	Negative	1 failed
3 - 5	G918E⌘	0.0008	0.003 (Latino)	2/0	72.2 (79–152)	NT	47.1 (16–41.2)	Negative	Negative	None
6	T956M	0.13	0.17 (Caucasian)	366/2	68 (90–180)	NT	56.8 (37–68)	38	1.02	1 failed
7	T956M	0.13	0.17 (Caucasian)	366/2	105 (75–175)	25 (14–40)	79.9 (37–68)	Negative	0.3	Allograft functioning well
8	L1207I	Novel	NT	Not reported/NA	NT	NT	NT	Negative	NT	Donor candidacy declined

Minor allele frequency (MAF) values were based on total and specific population frequency from Genome aggregation database (gnomAD).

**Laboratory tests showed characteristics of a TMA—anemia [hemoglobin, 7.7 g/dl (normal range, 12.0–15.0 g/dl); elevated LDH, 292 U/L (normal range, 100–250 U/L); low haptoglobin, 17 mg/dl (normal range, 30–200 mg/dl)]; thrombocytopenia [platelet count, 66 k/μl (normal range, 150–400 k/μl)], and AKI (creatinine, 8.94 mg/dl with baseline 1.5 mg/dl); schistocytes on peripheral smear (3–7/HPF).*

⌘Laboratory studies were consistent with a TMA—anemia [hemoglobin, 3.9 g/dl (normal range, 12–15.5 g/dl); elevated LDH, 1,723 U/L (normal range, 140–280 U/L); undetectable haptoglobin, <30 mg/dl (normal range, 50–220 mg/dl)]; thrombocytopenia [platelet count, 71 k/μl (normal range, 150–450 k/μl)], and AKI (creatinine, 7.0 mg/dl with baseline 0.7 mg/dl); and schistocytes on peripheral smear (3–4/HPF). NT, not tested. I, isoleucine; V, valine; L, leucine; G, glycine; E, glutamic acid; T, threonine; M, methionine.

FIGURE 3 | Amino acid sequence alignment of complement control protein (CCP) repeats in FH. Sequence alignments are generated with NCBI protein BLAST software based on position-specific scoring matrix (PSSM). Red to blue color scale represents the degree of conservation; red represents highly conserved residues; blue represents less conserved residues. Upper-case residues are aligned, lower-case residues are unaligned. Mutated residues of FH in the cohort are indicated with arrows. Two disulfide bonds form in each CCP, one between cysteine I and III and the other between cysteine II and IV (red and highlighted).

Iowa for the C3b deposition and sheep red blood cell hemolytic assays. The C3b deposition assay was abnormal for I372V (40–60% C3b deposition detected; reference range, negative, MORL, Iowa) and the sheep hemolytic assay was abnormal for I453L (20–40% hemolysis detected; normal <3%, MORL, Iowa).

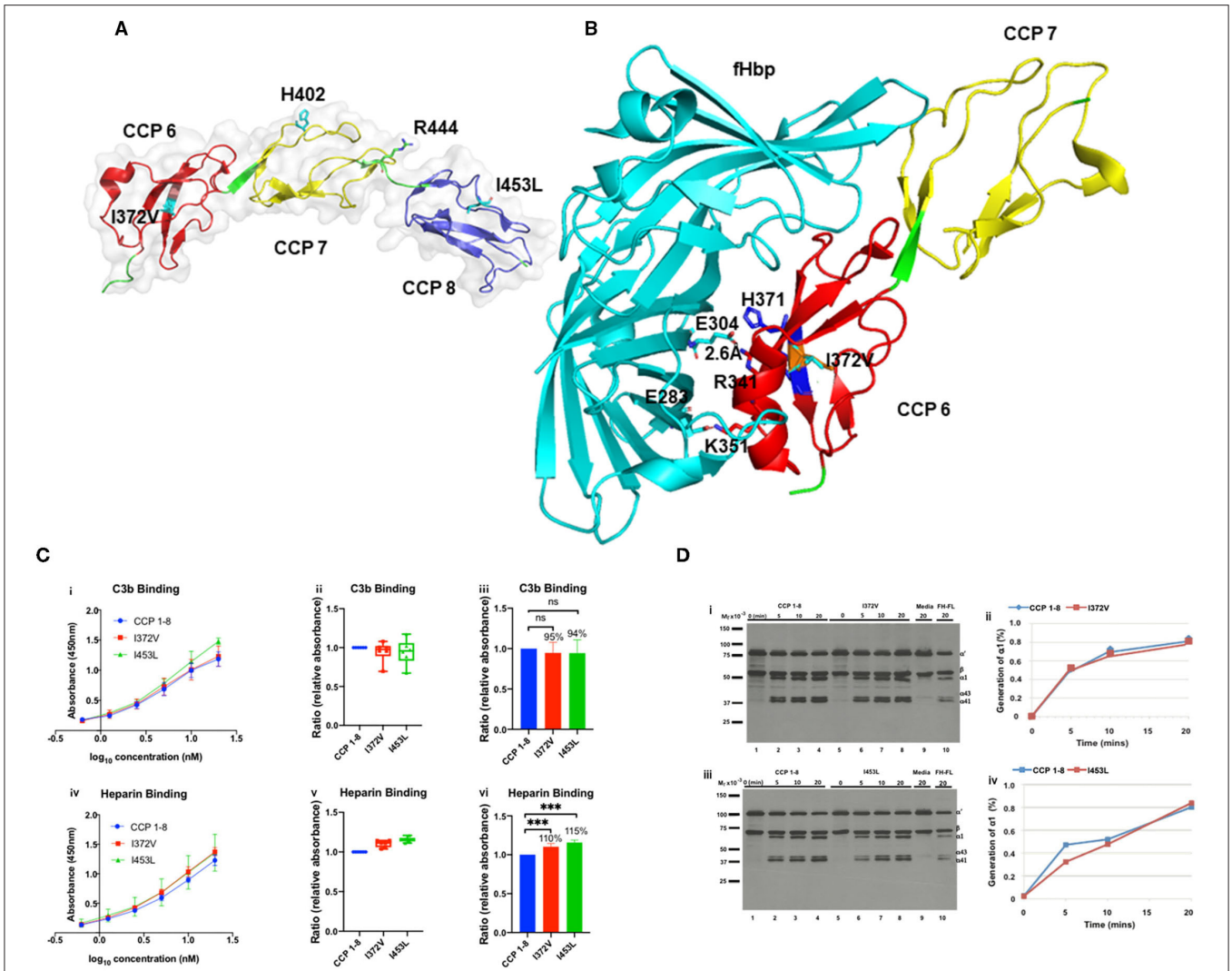

FIGURE 4 | Structural analysis of I372V and I453L **(A)** The overall structure of FH CCP 6–8 is shown in a cartoon representation (PDBID:2UWN) (20). CCP 6 is in red, CCP 7 in yellow, and CCP 8 in blue. H402 and the two rare variants, I372V and I453L, are in cyan. Crystallographic structural data indicates that CCP 6–8 contain four glycosaminoglycan (GAG) binding sites (20). The primary binding site is H402 (20); the other three putative binding sites are H360 (not shown) in CCP 6, R341 in CCP 6 (in **B**), and R444 in CCP 7 (colored green at the linker between CCP 7 and CCP 8). **(B)** Interface structure of FH CCP 6–7 in complex with FH binding protein (fHbp) derived from *Neisseria meningitidis* (PDBID: 2W80) (21). Side chains from both proteins involved in forming salt bridges across the interaction surface are shown. The putative heparin binding site is composed of H337 (not shown), R341 and H371 in CCP 6 (21). E304 and R341 form a salt bridge over a distance of approximately 2.6Å. E304 and E283 in cyan from fHbP; R341 and K351 from CCP 6 in red; the variant I372V in orange is flanked by H371 and H373 in blue. Structures are prepared using PyMol. Functional analysis of I372V and I453L **(C)** (i, iv) Absorbance is plotted against logarithmic protein concentrations. (ii, v) Box-and-whisker plots demonstrating C3b and heparin binding (by ELISA) of I372V and I453L compared to WT. Relative absorbance (RA) is computed as absorbance of the variant divided by absorbance of the WT. Bottom of the box shows the 25th percentile, the line within the box indicates the median, and the top of the box shows the 75th percentile. (iii, vi) Representation of C3b and heparin binding of I372V and I453L compared to WT using bar graphs. For C3b binding the *P*-value for the percentage differences of I372V and I453L compared to WT were 0.69 and 0.65, respectively. For heparin binding, the *P*-value for the percentage differences of I372V and I453L compared to WT were both <0.001. Data represent three separate experiments with bars corresponding to SEM (standard error of mean). ns, no significant difference; ***$P < 0.001$. **(D)** (i, iii) Fluid-phase C3b cofactor activity (CA) of I372V and I453L assessed by the cleavage of purified C3b to iC3b compared to WT. The percentage of alpha chain remaining and the generation of α1 indicates the cleavage of C3b to iC3b. A kinetic analysis of CA was conducted at 0, 5, 10, and 20 min. Cleavage rate is measured by densitometric analysis of the generation of α1 relative to the β chain. Representative Western blot is shown. (ii, iv) Densitometric quantification of the Western blot. Lane 9 represents a negative control employing concentrated supernatant in the presence of FI but absence of FH. Lane 10 is a positive control using full-length FH (FH-FL). Upon comparison to WT FH, there is no difference in CA of I372V and I453L.

Implications

Patients 1 and 2 represent examples of post-transplant TMA in patients with SLE in which a rare complement genetic variant was identified. This clinical scenario is challenging for clinicians to determine if such patients have a primary complement-mediated TMA or a secondary SLE-associated or a transplant-related TMA or a combination of the above. Our analyses demonstrate that the variants I372V and I453L are produced

TABLE 2 | Summary of the functional analyses for *CFH* variants.

Patient (s)	Variant	Location	Recombinant secretion (μg/ml)	C3b binding	Heparin binding	Cofactor activity	Cell-surface regulation using patient serum	ACMG interpretation	Modified interpretation (based on functional and structural analysis)
1	I372V	CCP 6	Comparable to WT	N	N	N	Defective (C3b deposition assay)	VUS	Deleterious
2	I453L	CCP 8	Comparable to WT	N	N	N	Defective (sheep red cell hemolytic assay)	VUS	Deleterious
3, 4	G918E	CCP 15	Decreased	ND	ND	__	Not done	VUS	Deleterious
3, 4	G918E/N1050Y	CCP 15/18	Decreased	ND	ND	__	Not done	VUS	Deleterious
6	T956M/E936D	CCP 16	Comparable to WT	N	N	__	Not done	VUS	Normal function
7	T956M	CCP 16	Comparable to WT	MD	N	__	Not done	VUS	Likely benign
8	L1207I	CCP20	Comparable to WT	N	N	__	Not done	VUS	Normal function

Secretion of I372V (5.9 μg/ml) and I453L (6.2 μg/ml) was comparable to WT CCP1–8 (4.0 μg/ml) ($P > 0.05$, SEM 1.15) as measured by ELISA. C3b binding and cofactor activity were normal but cell surface regulation was defective for both I372V and I453L. Variant G918E was not secreted. Secretion of SNP N1050Y (4.1 μg/ml) was comparable to WT CCP 15–20 (3.6 μg/ml) ($P < 0.05$, SEM 0.1). Secretion of G918E/N1050Y was markedly reduced compared to WT. Secretion of T956M (2.6 μg/ml) and T956M/E936D (3.5 μg/ml) were comparable with WT CCP 15–20 (3.6 μg/ml) ($P = 0.7855$, SEM 0.1). Secretion of L1207I was comparable to WT CCP 18–20 (2 μg/ml) ($P > 0.05$, SEM 0.16). ACMG, American College of Medical Genetics; VUS, variant of uncertain significance. Patient 5 did not undergo genetic testing due to financial/insurance issues. N, normal; ND, not done; MD, marginally decreased. I, isoleucine; V, valine; L, leucine; G, glycine; E, glutamic acid; T, threonine; M, methionine.

normally and thus have no quantitative defect but are deleterious due to a qualitative defect based on the distinctly abnormal serum-based assays. The structural analysis also supports a likely deleterious effect.

However, given that the recombinant FH protein demonstrated normal results but serum-based assay revealed impaired function in the C3b deposition or the sheep red blood cell hemolytic assays led us to consider other possibilities. The question was raised that elevated FHR levels in the serum might be the cause of impaired FH function of complement regulation. To address this issue, we re-examined the next generation sequencing data which included CFHR1, CFHR2, CFHR3, CFHR4, and CFHR5 genes. Additionally, CFHR3-CFHR1 copy number was assessed. Neither patient carried a variant in the CFHR genes. A heterozygous CFHR3-1 deletion was identified in patient carrying the variant I453L but not in patient carrying the variant I372V. Since altered FHR levels have not been associated with a heterozygous CFHR3-1 deletion, we think that it is less likely that the patients have elevated FHR levels but this possibility cannot entirely be ruled out.

To summarize, these two patients did not develop TMA in the native kidney but developed an early and aggressive disease in the allograft in the presence of a trigger(s) that included transplant surgery and/or rejection. Overall, these data help to clarify that the TMA in these two patients is likely complement-mediated. Therefore, in both cases it was recommended that eculizumab be administered at the time of the next kidney transplant.

Familial Postpartum TMA Syndrome With Multiple Genetic Variants

Clinical History

Patient 3 (Sister 1, Proband)

In 2015, a 25-year-old Hispanic female presented 10 days after an uncomplicated vaginal delivery with laboratory results consistent with a TMA (**Table 1**). Treatment with prednisone and plasma exchange (PE) was unsuccessful and HD was initiated. ADAMTS13 activity and APL Abs were normal. Eculizumab was started after kidney biopsy confirmed a TMA (not shown). Hemodialysis was discontinued after 8 weeks. Two months later, she developed flash pulmonary edema due to acute mitral valve regurgitation secondary to an anterior leaflet perforation, which necessitated a mitral valve replacement. The patient is currently doing well with stable kidney function. In retrospect, the proband's two sisters had similar clinical presentations.

Patient 4 (Sister 2)

In 2014, this 24-year-old presented 7 days postpartum after an uncomplicated vaginal delivery with anemia, thrombocytopenia, and AKI. She was unsuccessfully treated with PE and HD had to be initiated. The proband diagnosis was established 1 year later, leading to aHUS being considered in sister 2 and eculizumab was initiated. Her creatinine (Cr) initially stabilized at 2–2.5 mg/dl and was 0.9 mg/dl in December 2020. However, she did not continue eculizumab due to health insurance issues. Since discontinuation of eculizumab, the renal function has remained stable, but she has developed hematuria and proteinuria (1.2 g).

Patient 5 (Sister 3)

In 2006, this 18-year-old presented 7 days postpartum after an uncomplicated vaginal delivery with anemia, thrombocytopenia, and AKI. Similar to her sisters, she did not respond to PE and HD was initiated. The course was complicated by heart failure due to severe mitral and tricuspid regurgitation. She was not treated with eculizumab as aHUS was not considered until after the diagnosis had been made in her sisters (8 years later). She currently remains on dialysis.

Family history in this kindred is significant for a paternal grandmother who lost 10 of 12 pregnancies due to miscarriages, and a paternal aunt who is currently on dialysis

(**Supplementary Figure 2**). No medical history is available for the father. The mother delivered five healthy children (the three sisters mentioned above and two brothers) with no postpartum complications.

Genetic Analysis

The proband was a compound heterozygote for two non-synonymous variants in *CFH*, G918E, and N1050Y (**Table 1**; **Supplementary Table 3**). The G918E variant is rare being identified in two individuals in gnomAD, one each of European and Latino ancestry, and has also been reported in a patient with a TMA (23). The G918 residue is highly conserved across species and *in silico* predictions point to a deleterious effect of the G918E variant. Due primarily to a lack of *in vitro* studies, it was classified as a VUS.

The N1050Y SNP occurs at a position that is not highly conserved and *in silico* analyses yield inconsistent predictions. This variant has been associated with reduced AMD risk (24) and is not enriched in aHUS (25, 26). Based on these criteria, the N1050Y variant was classified as benign.

A heterozygous splice-site variant (c.287-2A>G) was identified in the *MCP* (*CD46*) (27). *In vitro* studies are consistent with an exon skipping effect predicted to result in protein truncation (27, 28). This variant has been identified in aHUS, and is classified as a pathogenic variant based on functional, computational and population evidence (2, 29, 30).

A heterozygous missense variant, K441R, in *CFI* was also identified and previously described as likely benign (31). Sister 2 carries the same genetic variants in *CFH* and *MCP* as the proband but does not carry the variant in *CFI*. Unfortunately sister 3 did not undergo genetic testing.

Structural Analysis

G918 is located in CCP 15 between the highly conserved C915 and W920 residues (**Figure 3**). Substitution of G with E introduces a negatively charged side chain at the main surface loop between β-strands 6 and 7 (**Figure 5A**). This alteration could generate side chain clashes between E918 and I868 that perturb the conformation of the adjacent C870–C915 disulfide bond and thereby disrupt FH protein folding.

N1050 is located in CCP 18 (**Figure 3**), close to the interdomain linker between CCP 17 and CCP 18, which could alter the inter-CCP domain arrangements. Because Y has a larger relative size than N, its presence in the variant may modulate the overall folded-back CCP domain structure but should not affect FH stability.

Antigenic and Functional Analyses

Factor H and FI antigenic levels in the proband were normal, but MCP expression was low (**Table 1**). Secretion of the recombinantly produced variant protein G918E was undetectable (**Figure 2B**; **Table 2**). By contrast, the recombinant N1050Y SNP displayed higher secretion than WT (**Table 2**). We produced the combined variant G918E/N1050Y and showed that the secretion was markedly reduced (**Figure 2B**). We also recombinantly produced the CFI variant K441R which displayed normal secretion and no defect in cofactor activity (not shown).

Implications

These cases represent pregnancy-associated TMA in a family that carries genetic variants in multiple complement proteins (FH, MCP, and FI). The MCP mutation is pathogenic given the low expression in our patient and having been reported previously as having low expression on granulocytes (27). Our structural and functional data establish that the *CFH* variant G918E is also deleterious because it abolishes FH protein expression. The proband though had a normal FH level. We speculate that in our patient this may be due to enhanced protein production by the N1050Y SNP, likely consistent with N1050Y location on the other allele. This hypothesis is supported by the fact that another patient carrying G918E had a reduced FH level of 168 mg/l (normal range 180–420 mg/l; personal communication, Richard Smith, Iowa).

These results indicate that TMA etiology in these patients is complement-mediated based on defective *CFH* and *MCP* variants. The risk of recurrent TMA after a kidney transplant in patients carrying an MCP mutation is low since the allograft comes with a normal MCP. However, since the two sisters also harbor the dysfunctional *CFH* mutation, they are at higher risk for recurrent disease.

We propose that complement dysregulation may have caused multiple miscarriages in the paternal grandmother and ESRD in the paternal aunt. The presentation of acute heart failure (AHF) due to valvular disease in two sisters is unusual and has not been reported previously. We propose that the mechanism underlying AHF is TMA-associated vasculitis with thrombus formation leading to valvular dysfunction.

Our work highlights a unique postpartum aHUS presentation with a remarkable phenotype involving the renal and cardiac systems and illustrates the putative role of multiple variants in disease etiology (33).

C3 Glomerulopathy Associated With and Without a Monoclonal Gammopathy

Clinical History

Patient 6

A 67-year-old Caucasian female with biopsy-proven C3GN in the setting of a monoclonal gammopathy (elevated IgG kappa on serum protein electrophoresis and immunofixation) underwent a DDKT in 2016. She had an elevated FH autoantibody and a C3 nephritic factor (C3Nef; 1.02, normal range 0.0–0.26). Prior to transplant, she was treated with bortezemib, cyclophosphamide, and dexamethasone, which reduced the M-protein (≥90%) and led to negative tests for both autoantibodies. She developed recurrent C3GN in 2018 (**Supplementary Figure 3**). A bone marrow biopsy revealed involvement by the previously diagnosed plasma cell neoplasm. A year later the patient died secondary to refractory congestive heart failure.

Patient 7

A 28-year-old Caucasian male with biopsy-proven C3GN underwent a living unrelated kidney transplant from his wife in Feb 2020. He had an elevated C3Nef (0.3, normal range 0–0.26, Blood Center of Wisconsin) associated with a high Bb level (2.7 mg/l, normal <1.2 mg/l) and an elevated soluble C5b-9 (1.51

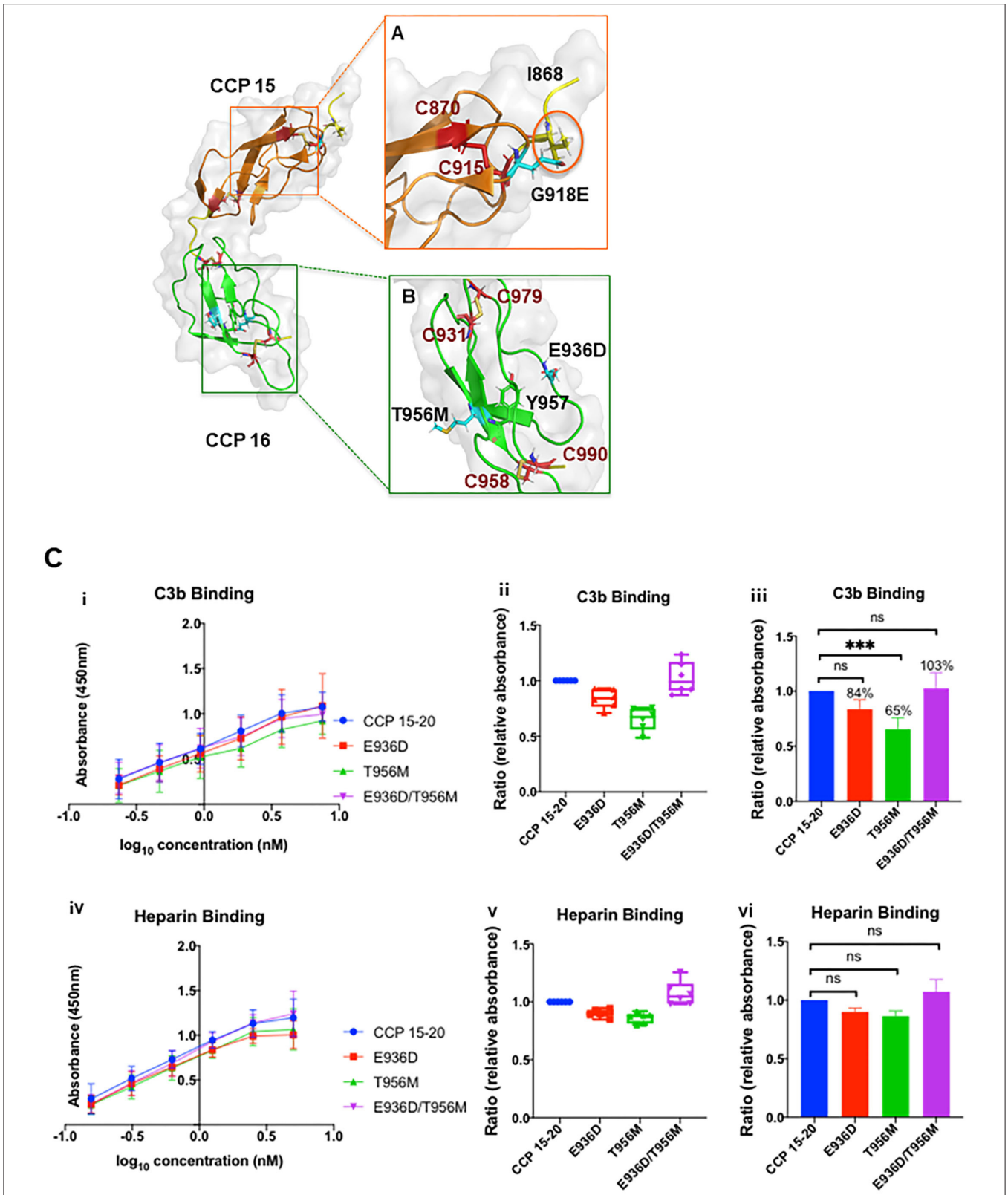

FIGURE 5 | Structural analysis of G918E, E936D, and T956M. Nuclear magnetic resonance spectroscopy solution structure of CCP 15–16 (PDB ID: 1HFH) (32). **(A)** The highest frequency rotamer of the G918E side chain and its clashing residue I868 is highlighted in the orange circle. **(B)** The highest frequency rotamer of the

(Continued)

FIGURE 5 | T956M and E936D side chains is highlighted in cyan. C958 and C990 form a disulfide bond next to the Y957 residue to stabilize the CCP 16 tertiary structure. E936D and T956M are located on the protein surface, spatially distant from the disulfide bond. CCP 15 (orange); CCP 16 (green); linker between CCP 15 and 16 (yellow); cysteine side chains involved in disulfide bond formation (red); G918E, E936D, and T956M variants (cyan sticks, zoomed and reoriented in inset box). Functional analysis of T956M and E936D. **(C)** (i, iv) Absorbance is plotted against logarithmic protein concentrations. (ii, v) Box-and-whisker plots demonstrating C3b and heparin binding (by ELISA) of E936D, T956M, and combined variant E936D/T956M compared to WT. (iii, vi) Representation of C3b and heparin binding of E936D, T956M, and E936D/T956M compared to WT using bar graphs. Compared to WT there was no difference in heparin binding for E936D, T956M, or E936D/T956M. T956M showed ∼35% decrease in C3b binding compared to WT ($P < 0.001$). Data represent three separate experiments with bars corresponding to SEM. $^{***}P < 0.001$.

mg/l, normal <0.3 mg/l). The alternative pathway complement functional assay (AH50) was low (72%, normal 75–170) as well as the properdin level (7.5 mg/l, normal 10–33 mg/l). A monoclonal gammopathy was not detected (**Table 1**). There was no family history of kidney disease. At last follow-up in March 2021, Cr was stable at 1.2 mg/dl with no proteinuria.

Genetic Analysis

A rare heterozygous variant T956M was identified in patients 6 and 7 (**Table 1**). This variant is located at a position that is not highly conserved but has been reported previously in patients with aHUS (34–36). Most *in silico* tools predict that this variant is tolerated; however, MutationAssessor and Polphen2-Hdiv predict a damaging effect. It was classified as a VUS due to a lack of functional studies. Patient 6 also carried a SNP, E936D, which is common across multiple populations and is associated with a predisposition to aHUS (**Supplementary Table 3**) (37). *In silico* tools however predict that E936D is benign.

Structural Analysis

The T956 residue in CCP 16 is less conserved and adjacent to the highly conserved Y957 residue (**Figure 3**). The hydrophobic Y957 side chain is packed in the CCP 16 interior, whereas the hydrophilic T956 side chain is exposed on the protein surface (**Figure 5B**). The substitution of hydrophilic T with the long hydrophobic side chain M in the T956M variant may perturb the beta sheet orientation, and therefore alter CCP 16 folding.

The E936 residue in CCP 16 is less conserved in multiple CCP sequence alignments (**Figure 3**). E and D are both negatively charged hydrophilic amino acids in the E936D variant, and the substitution is highly tolerated without interfering with protein folding (**Figure 5B**).

Antigenic and Functional Analysis

Factor H antigenic levels in both patients were normal (**Table 1**). Secretion of the recombinantly produced T956M variant was comparable to that of WT (**Figure 2**; **Table 2**). The variant had slightly reduced affinity for C3b binding compared to WT, but unchanged affinity for heparin binding (**Figure 5C**). Defective C3b binding was corrected with the double mutant E936D/T956M.

Implications

These two patients with C3GN carry a genetic variant (T956M) and acquired defects in the form of autoantibodies (C3Nef and/or FH). Our analyses demonstrate that T956M may have a modest functional defect due to ∼30% decrease in C3b binding. However, the function improved when combined as variant E936D/T956M (**Figure 5C**). The T956M variant was also previously reported in an aHUS patient and functional data showed no defect (36). Thus, while the structural analysis proposes a possible deleterious effect, functional analysis by us and others establish that T956M is likely benign. Therefore, the development of autoantibodies (FH and C3Nef) in patient 6 is most likely related to the underlying monoclonal gammopathy, a well-known association of C3G (38). Patient 7 persistently harbors an elevated C3Nef and an abnormal complement biomarker profile (due to an unclear etiology) despite being on immunosuppression and thus is at risk for recurrent C3GN.

Living Donor Kidney Transplantation in aHUS

Clinical History

Patient 8

A 63-year-old Caucasian male was evaluated as a kidney donor for his 36-year-old son. The son had developed ESRD at age 33 and was given a diagnosis of possible aHUS. He was initially treated with plasmapheresis and subsequently switched to eculizumab. The son underwent genetic testing which identified a heterozygous CFHR3-1 deletion. The father underwent genetic testing as part of donor evaluation work-up. This case was referred to us from an outside hospital to assist with genetic interpretation of the VUS identified in the father as described below.

Genetic Analysis

A novel heterozygous missense variant L1207I was identified. This variant is located within a poorly conserved nucleotide position in CCP 20 (**Figure 3**) (39). *In silico* functional prediction indicated a tolerated effect of this alteration. This variant though was categorized as a VUS due to the paucity of functional studies and lack of other pathogenicity supporting evidence. Two SNPs (I1059T and Q1143E) and a homozygous CFHR3-1 deletion were also identified. The father or son did not carry FH-autoantibodies.

Structural Analysis

CCP 20 interacts with the C3d cleavage fragment of C3b and GAGs (14). C3d binds electrostatically with CCP 20. The interacting surface consists of a positively charged pole formed by R1203, K1230, and R1231 from CCP 20, and a negatively charged cleft formed by E160, D163, and D292 from the C3d moiety of C3b (**Figure 6A**) (40). CCP 20 has a GAG binding site that is formed by a hydrophobic pocket. The key amino acids identified in this region are L1181, R1182, W1183, S1191, E1195, S1196, E1198, and R1215 (**Figure 6B**)

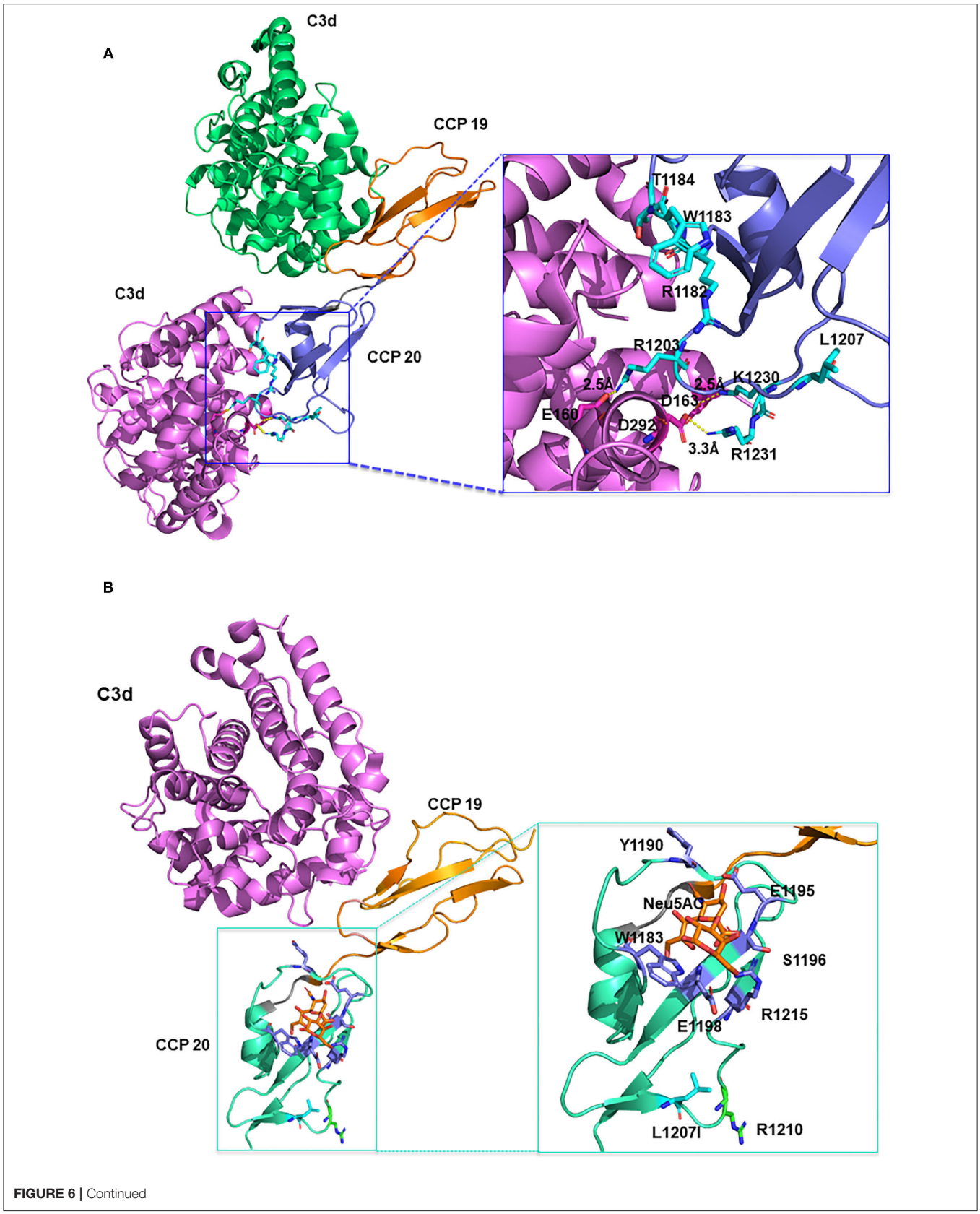

FIGURE 6 | Continued

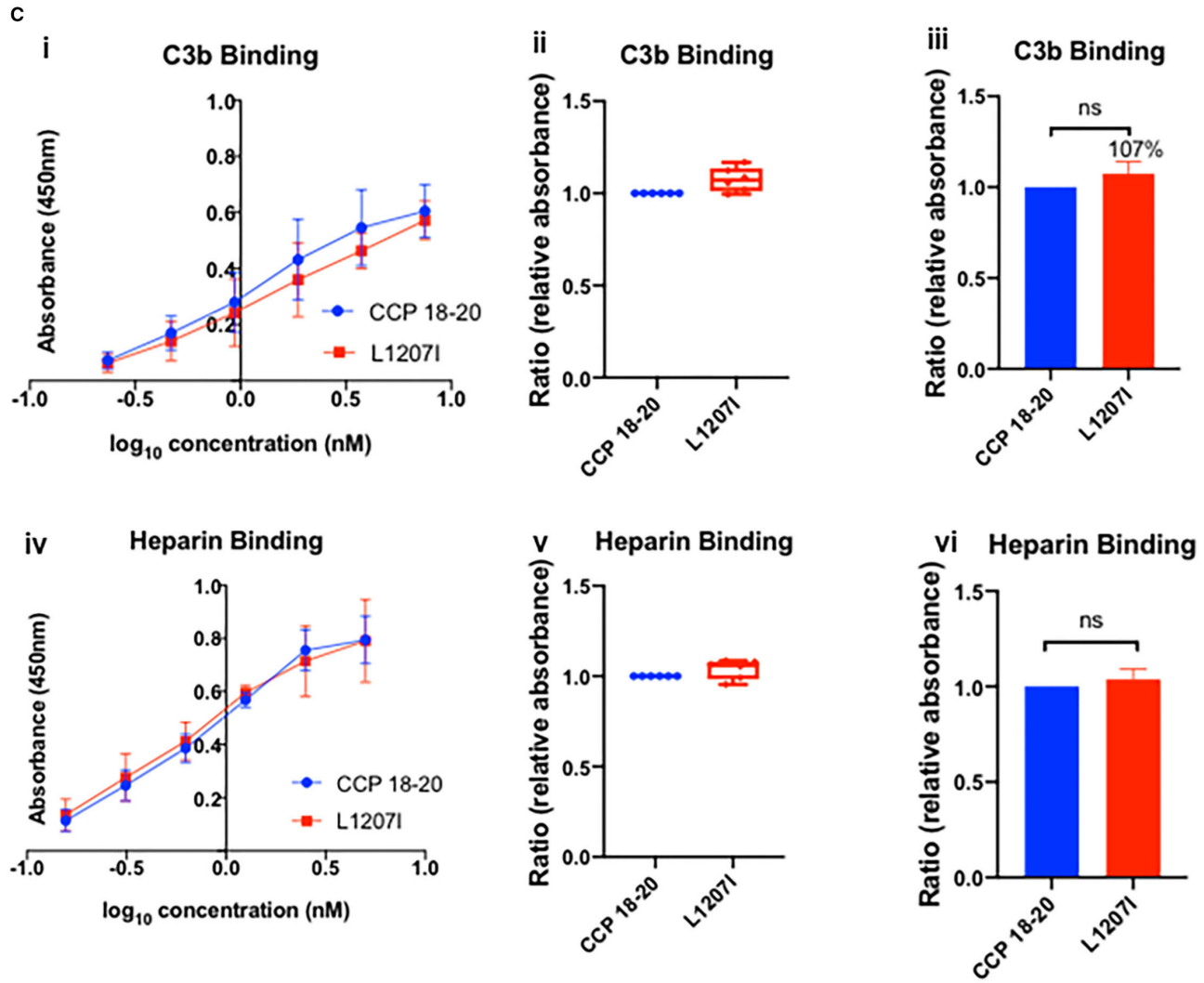

FIGURE 6 | Structural analysis of L1207I. **(A)** Structure of the CCP 19–20:C3d complex containing one molecule of CCP 19–20 and two molecules of C3d (PDB ID 2XQW, green and purple) (40). Residues located at the CCP 20 interface are annotated and shown as cyan sticks. Residues located at the C3d interface are shown as magenta sticks. Close-up view of the CCP 20-C3d interface is shown and reoriented in the inset box. R1182, W1183, and T1184 mutations shown as cyan sticks were reported in aHUS cases (40). Functional study of W1183L demonstrated impaired interaction with surface-bound C3b (14). E160 and R1203, D163 and K1230, D292 and R1231 form salt bridges with distances of approximately 2.5, 2.5, and 3.3 Å, respectively. L1207 (in cyan) is located away from the C3d-CCP 20 interaction surface. **(B)** Sialic acid binding site on complement CCP 20 (PDB ID 4ONT) (41)—C3d (magenta); CCP 19 (orange); CCP 20 (green); α-N-acetylneuraminic acid (Neu5AC, orange stick). L1207 (cyan) is spatially distant from the binding surface between Neu5AC and CCP 20. R1210 mutations (green sticks) have been reported in aHUS. (42). Functional analysis of L1207I **(C)** (i, iv) Absorbance is plotted against logarithmic protein concentrations. (ii, v) Box-and-whisker plots demonstrating C3b and heparin binding (by ELISA) of L1207I compared to WT. (iii, vi) Representation of C3b and heparin binding of L1207I compared to WT using bar graphs. There is no difference in C3b or heparin binding for L1207I compared to WT. Data represent three separate experiments, bars correspond to SEM.

(14). L1207 does not appear to directly coordinate either C3d or GAG binding. L and I are two hydrophobic and highly interchangeable amino acids, and the substitution of L to I likely does not alter the overall conformation of the C3b and heparin binding sites.

Antigenic and Functional Analysis

Secretion of the recombinantly produced variant protein in the supernatant was normal compared to that of WT (WT, 1.1 μg/ml; L1207I, 1.07 μg/ml) (**Figure 2C**). Functional studies confirmed that the variant had no significant effect on either C3b or heparin binding compared to that of WT ($P > 0.05$) (**Figure 6C**). Functional analysis of the SNPs (I1059T and Q1143E) was also performed and demonstrated no defect (**Supplementary Figure 4**).

Implications

This case is challenging because it involves a living donor transplant for a father-son pair. The son (potential recipient) has a presumptive diagnosis of aHUS with no identified

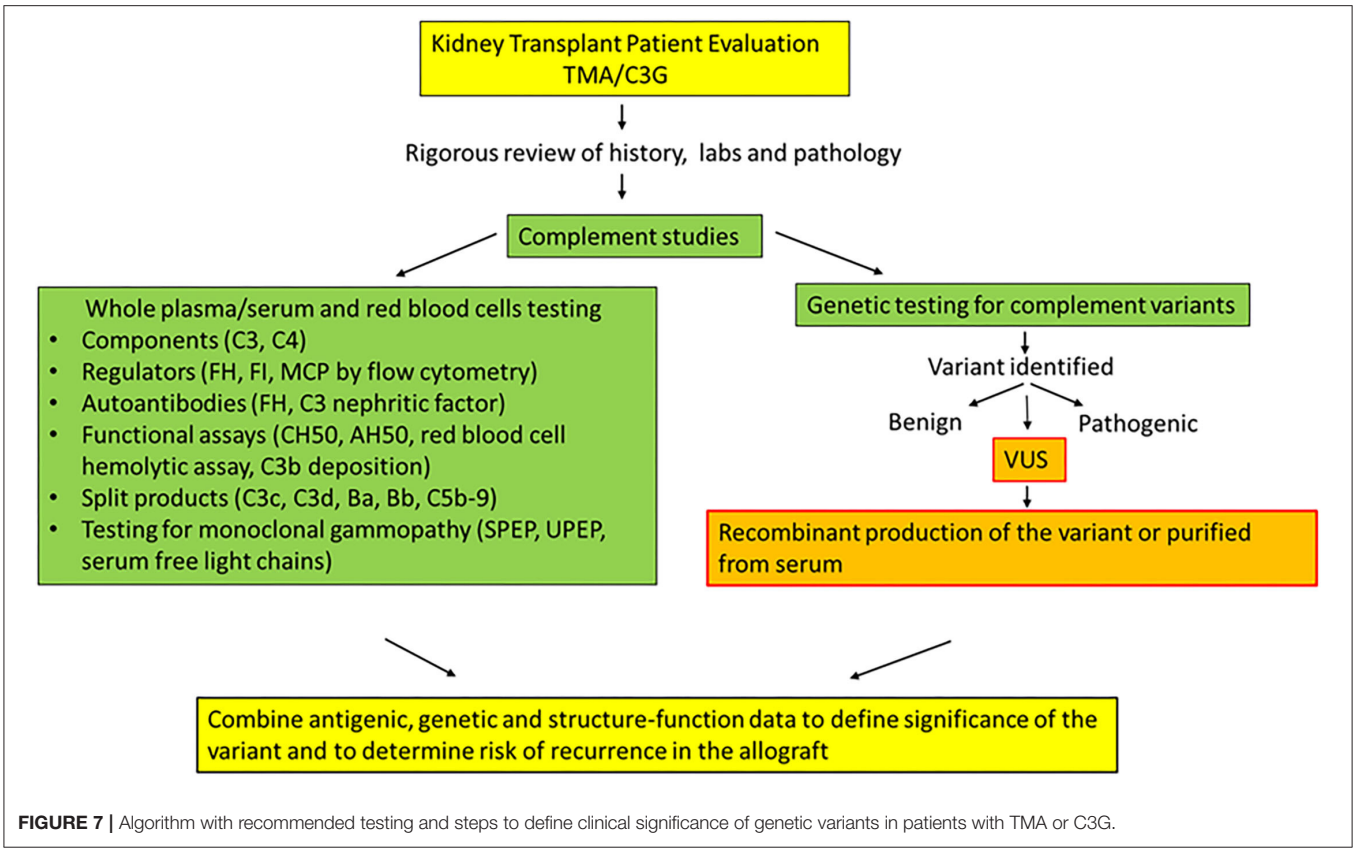

FIGURE 7 | Algorithm with recommended testing and steps to define clinical significance of genetic variants in patients with TMA or C3G.

genetic variants, whereas the healthy father (potential donor) has a rare genetic variant (L1207I) and SNPs in *CFH*. Our analyses showed that L1207I is not functionally or structurally deleterious and probably explains why the father did not develop kidney disease. In this case, donor candidacy was declined since functional analysis of the VUS was not available at the time of transplantation. More importantly, a definitive diagnosis of aHUS and its etiology in the son remains unknown.

Living-related donor kidney transplant is generally contraindicated for patients with aHUS (43) because a nephrectomy may trigger TMA in the genetically susceptible donor. Although donor genetic analysis may be performed, some recipients have more than one mutation and genetic testing does not reveal a variant in the complement gene in approximately one-third of patients with aHUS (such as in this case). We may consider living-related donor transplants in patients who carry a known pathogenic mutation in a complement protein that causes disease in the patient if the donor tests negative for that mutation. The recipient and living donor should participate in the decision-making process after they understand the risks and benefits.

DISCUSSION

We describe clinical cases of eight patients with a TMA or C3G from our kidney transplant cohort. In each case, we were faced with the challenge of determining the disease etiology and recurrence risk because of the presence of confounding risk factors in their clinical history. Moreover, these patients carried genetic variants in *CFH* that were classified as a VUS. Since there was a lack of information in the literature about the functional impact of these variants, we employed an integrative approach by utilizing the clinical, genetic, antigenic and structural data in addition to the functional analyses, to further define the implications for each variant (5).

Our work highlights a multi-modality approach that substantially facilitated: (i) Establishing the diagnosis of a complement-mediated TMA in a complex clinical setting (lupus) with multiple triggers (Patients 1 and 2) (ii) Defining the role of multiple deleterious variants in a unique postpartum TMA presentation featuring a three-sister kindred with multiple organ system involvement (Patients 3, 4, and 5). (iii) Illustrating the prominent role of acquired factors (monoclonal gammopathy, C3 nephritic factor, and FH autoantibody) in C3G even though a rare variant was identified but demonstrated normal function (Patients 6 and 7) (iv) Formulating an informed therapeutic decision about a potential living donor transplantation in the setting of a TMA (Patient 8).

Our results point out potential problems in using the ACMG guidelines to interpret variants. These guidelines are designed for Mendelian diseases and heavily weigh population frequency. Consequently, despite functional data demonstrating

no defect, the interpretation for many of the genetic variants described in our cases remained a VUS primarily due to the rarity of the variant. Based on these results, we suggest that modifications to the ACMG framework for diseases with variable penetrance, such as aHUS, be considered. For combined variants, we speculated but could not confirm if they were present on the same or different alleles. One way to further address this issue is to test family members to determine whether variants are shared by other affected and unaffected relatives. In our cases, the family samples were not available for sequencing.

There are two quite informative studies on *CFH* variants in aHUS and C3G by Merinero et al. that are particularly worth noting here. In the first one, the authors report functional characterization of *CFH* variants using FH purified from plasma samples of FH Y402H heterozygous carriers (36). This was an elegant approach to obtaining purified full-length FH which was available for 7 of 12 variants. While our manuscript was being prepared for submission, a second comprehensive study reported a functional assessment for 105 *CFH* variants associated with aHUS (44). In this study, full length FH was prepared recombinantly by ThermoFisher Scientific (Regensburg, Germany). Of note, four of the five VUS we describe here have not been reported previously including in this recent publication. The one overlapping variant, T956M, demonstrated similar results to our data. Also, the SNPs (E936D, N1050Y, I1059T, Q1143E) have been reported and results of our functional data are comparable (**Supplementary Figure 4**) (44). Additionally, we demonstrate that the SNP E936D may have a potentially protective role since it helped improve the C3b binding for rare variant, T956M. We used FH fragments which have been shown by us (16) and others (45–47) to be highly predictive of the results obtained with full-length protein (36, 44). Nevertheless, the availability of commercially available full-length recombinant FH and the serum-based assays is an advance that should be taken advantage of.

In summary, there are challenges in reconciling genetic data with clinical management. The strategy of recombinant protein production followed by systematic antigenic, functional, and structural assessment remains the gold standard (**Figure 7**). We predict that such functional assessments will become more widely available in the future and will be critical to address knowledge gaps and identify the etiology and pathogenic mechanisms in patients with complement-mediated kidney diseases, especially in a complex clinical setting of kidney transplantation. The ongoing development of complement biomarkers and novel assays will provide clinicians with the tools to stratify patients for targeted therapy and adapt precision medicine for genetic kidney diseases.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are publicly available. These data can be found here: ncbi.nlm.nih.gov/clinvar/, SCV001905506–SCV001905512.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board Washington University School of Medicine in St. Louis, MO. The patients/participants provided their written informed consent to participate in this study. Written informed consent was obtained from the individual(s) for the publication of any potentially identifiable images or data included in this article.

AUTHOR CONTRIBUTIONS

ZR, AJ, and JA conceived and designed the experiments, analyzed the data, and interpreted results. ZR performed the experiments. ZR and SP conducted the structural analysis. LL-G classified variants in accordance with ACMG 2015 criteria. ZR prepared the figures and drafted the manuscript. ZR, SP, LL-G, JA, and AJ edited the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.775280/full#supplementary-material

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Clinical Outcomes of Patients With Primary Membranous Nephropathy and Subnephrotic Proteinuria

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Objectives: To update the information about the prognosis of patients with primary membranous nephropathy (MN) and subnephrotic proteinuria and identify the relevant predictors.

Methods: In total, 474 cases of biopsy-proven primary MN with at least 18 months of follow-up were reviewed to determine the outcomes of the subgroup of patients that presented with subnephrotic proteinuria. Clinical data included initial proteinuria and microhematuria, defined as the average proteinuria/microhematuria of the first 6 months during the course. Outcomes included partial remission (PR), complete remission (CR), nephrotic proteinuria progression, and kidney function progression, defined as ≥50% loss of kidney function or end-stage kidney disease.

Results: In total, 205 patients with primary MN and subnephrotic proteinuria at biopsy were eligible. During a median follow-up of 43 months, 200 (97.56%), 167 (81.46%), and 53 (25.85%) patients attained PR, CR, and nephrotic proteinuria progression, respectively. Only one patient (0.49%) progressed to the kidney function progression. By multivariate Cox hazards regression analyses, the initial proteinuria was identified as the independent predictor for PR, CR, and nephrotic proteinuria progression with adjusted hazard ratios (aHRs) of 0.67 (95% confidence interval, 0.56–0.80), 0.50 (95% CI, 0.40–0.63), and 2.97 (95% CI, 2.23–3.97), respectively. A higher level of initial microhematuria was also associated with an increased risk of nephrotic proteinuria progression. The corresponding aHR was 1.11 (95% CI, 1.05–1.17).

Conclusion: Among patients with primary MN and subnephrotic proteinuria, although the overall prognosis is excellent, dynamic detection and effective management of proteinuria remain important. In addition, initial microhematuria may be another predictor of nephrotic proteinuria progression.

Keywords: primary membranous nephropathy, subnephrotic proteinuria, clinical relapse, kidney function progression, remission

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INTRODUCTION

Primary membranous nephropathy (MN), an autoimmune glomerular disease, is one of the most common causes of primary nephrotic syndrome in adults. Approximately 20% of patients with primary MN present with subnephrotic range proteinuria (24 h urinary protein excretion <3.5 g/d), however, 61% later develop nephrotic range proteinuria (24 h urinary protein excretion ≥3.5 g/d), usually within the first year (1–7). There is also evidence to support the long-term benefit of persistent subnephrotic proteinuria with renal survival of >80–90% at 10 years (8). Recently, the independent relationships between several clinical features and long-term renal function decline have been increasingly noted in the overall population of primary MN, e.g., age, male gender, increased proteinuria during the course, decreased estimated glomerular filtration rate (eGFR) on presentation, high levels of phospholipase A_2 receptor (PLA2R) antibody after therapy, and C3 staining in the renal biopsy sample, etc. (2, 3, 5–7). However, among those with subnephrotic proteinuria, there was not sufficient data to describe the clinical prognosis and specific predictors.

Although current studies suggest these patients overall do well, the prognostic assessment of this subset of patients is controversial to some extent due to the lack of sufficient attention and data support. In the clinical practice, by virtue of the initial benign presentation and absence of specific predictive markers, some of these patients were not properly monitored and treated, putting them at the risk of developing nephrotic range protenuria and kidney function loss. To our knowledge, the study with the most sufficient sample size and follow-up was the cohort of Hladunewich et al. which indicated that, compared with patients with primary MN and persistent subnephrotic range proteinuria, those who subsequently attained nephrotic proteinuria showed a rate of kidney disease progression ∼4 times faster. However, the only baseline feature with statistical significance between the two groups was the level of proteinuria; besides, a few years have passed (7).

Glomerular microhematuria is one of the most common symptoms of glomerulonephritis. Recently, it is considered as a biomarker of activity in IgAN, ANCA-associated vasculitis, or lupus nephritis, and some data indicated that persistent microhematuria in IgAN is related to a greater risk of kidney disease progression (9–17). In primary MN, microhematuria is not uncommon and accounts for ∼50% of patients at presentation and 60% during the course (1, 2). However, based on the atypical nature of clinical characters and the absence of standardized methods to quantify (18, 19), little attention has been paid to the prognostic relevance of microhematuria in primary MN. Furthermore, Gutiérrez et al. suggested that the long-term outcomes in patients with biopsy-proven IgAN, isolated microhematuria, and minimal proteinuria at presentation are excellent (20). It is of great significance to systematically describe the prognosis of primary MN patients presenting with subnephrotic or minimal proteinuria and microhematuria at presentation.

In this retrospective cohort, we investigated the clinical course and outcomes among patients with primary MN and subneprotic range proteinuria. Longitudinal analyses were done to determine the corresponding prognostic factors and to quantify the strength of relevance.

METHODS

Study Population

We included in the present study patients admitted to the Department of Nephrology, Xijing Hospital (Xi'an, China) between October 1, 2015, and June 30, 2019, with biopsy-proven primary MN and subnephrotic proteinuria (24 h urinary protein excretion <3.5 g/d) at baseline, established as the time of kidney biopsy. Additional inclusive criteria included: a baseline eGFR of >15 ml/min/1.73 m^2, calculated according to the Chronic Kidney Disease Epidemiology Collaboration equation (21); at least 18 months of follow-up; and sufficient information on treatments and laboratory parameters for analyses. Patients with MN that associated with other diseases or exposures, such as infections, autoimmune diseases, malignancy, drugs/toxins, were diagnosed as secondary MN and excluded. Those with atypical MN (identified by kidney biopsy), or other concomitant glomerular diseases were also excluded. The study has been approved by the ethics committee of Xijing Hospital.

Data Collected

Patients were collected retrospectively, and their medical history, clinical data, and standard laboratory parameters were documented. The follow-up data were last updated on 31 December 2020. Demographic characteristics included age, gender, body mass index (BMI), comorbidities, and smoking status at the time of kidney biopsy. Initial and follow-up variables involved the assessment of blood pressure (BP), serum creatinine, serum albumin, microscopic analysis of urinary sediment, as well as 24 h proteinuria excretion. As for the microhematuria, we only recorded the results of urine sediment analyses, mainly of glomerular derived erythrocytes (dysmorphic erythrocytes >70%). The titers of serum anti-PLA2R antibody were tested using indirect immunofluorescence assays (IIFAs) and reported according to the fluorescence intensities and dilutions (1:10, 1:100, 1:1000) of the serum samples. The intensities of PLA2R and C3 staining in pathological specimens (using the IIFAs) were standardly reported and recorded as -, +, ++, + + +, or + + ++. The exposure to immunosuppressive (IS) agents (including corticosteroids, tacrolimus, and cyclophosphamide), statin class medications, and BP medications, including the angiotensin-converting enzyme inhibitors (ACEIs) or angiotensin receptor blockers (ARBs), were reported as intent to treat regardless of the duration of exposure.

Definitions and Outcomes

The follow-up time referred to the interval between kidney biopsy and the last outpatient visit, death, or end-stage renal disease (ESRD), whichever occurred first. The ESRD was defined by an eGFR value of <15 ml/min/1.73 m^2 or need of chronic dialysis. Hypertension was defined by systolic BP ≥ 140 mmHg, or diastolic BP ≥ 90 mmHg, or taking BP medications. Additionally, a high level of serum anti-PLA2R

antibody was defined by a titer of ≥1:100, and high intensity of PLA2R or C3 staining was defined as an intensity of ≥ ++ in the IIFAs. Due to the high variability of proteinuria and microhematuria assessment at a single time, initial proteinuria (initial microhematuria) referred to the average proteinuria (microhematuria) of the first 6-month block during follow-up. Initial persistent microhematuria was defined by an initial microhematuria of >5 red blood cell counts (RBCs)/high-power field (HPF).

As for patients with primary MN and subnephrotic proteinuria, partial remission (PR) was defined by a proteinuria value of <3.5 g/d plus a ≥50% reduction from its peak value, with stable kidney function. Complete remission (CR) was a proteinuria value of ≤0.3 g/d, along with normal serum albumin (≥3.5 g/dl) and stable kidney function. Nephrotic proteinuria progression was defined by an appearance of proteinuria ≥3.5 g/d. The primary outcomes were the PR, CR, and nephrotic proteinuria progression. The secondary outcome was kidney function progression, defined by a ≥50% decline in the eGFR or ESRD.

Statistical Analysis

Metric data were summarized as mean with standard deviation (SD) or median with interquartile range (IQR) according to its distribution. Categorical data were expressed as numbers with percentages. The included participants were classified into three groups (T1, T2, and T3) in reference to tertiles of initial proteinuria. The comparisons between groups were done using tests for trend, involving Cochran-Armitage trend test or Spearman's rank correlation test as appropriate. Clinical characteristics for never nephrotic and nephrotic proteinuria progression groups were compared using Student's *t*-test for normally distributed variables, Wilcoxon-rank sum test for variables with skewed distributions, and the chi-squared test for categorical variables.

Cumulative incidence rates of PR, CR, and nephrotic proteinuria progression were plotted and compared using Kaplan-Meier analyses and Log-rank tests. Univariate and multivariate Cox hazards regression analyses were done to identify independent prognostic factors and yield hazard ratios (HRs) with 95% confidence intervals (CIs). The variables with $P < 0.05$ in univariate analyses were included in multivariate analyses. For all the analyses, complete case methods were adopted, for which each analysis was restricted to participants with complete data for all factors in models. A two-sided $P < 0.05$ was considered statistically significant. SPSS version 22.0 (IBM, Chicago, IL, USA) and Stata version 15.0 (Stata Corporation, College Station, TX, USA) were used for statistical analyses.

RESULTS

In the entire cohort, among participants with primary MN, 27.12% (205 of 756) presented with subnephrotic range proteinuria, and preserved kidney function were collected (**Figure 1**). Their baseline and follow-up data are shown in **Table 1**. The median age was 48 years (IQR, 31–55 years), and 103 (50.24%) patients were males. The mean BMI was 24.12 kg/m^2 (SD, 3.82 kg/m^2). In total, 64 (31.22%) patients were hypertensive, 15 (7.32%) patients had diabetes, and 38 (18.54%) were smokers. All patients had normal kidney function [the mean serum creatinine was 0.85 mg/dl (SD, 0.16 mg/dl), and the median eGFR was 96.47 ml/min/1.73 m^2 (SD, 19.08 ml/min/1.73 m^2)]. The median proteinuria was 1.62 g/d (IQR, 0.90–2.22 g/d), median microhematuria was 3 RBCs/HPF (IQR, 1–8 RBCs/HPF), and the mean serum albumin was 3.22 g/dl (SD, 0.73 g/dl). Additionally, 73 (40.78%), 110 (65.87%), and 24 (13.79%) presented with a high level of serum anti-PLA2R antibody, high intensity of PLA2R staining, and high intensity of C3 staining, respectively. In the primary analysis, data were missing for 7.32, 12.68, 18.54, and 15.12% of patients for BMI, serum anti-PLA2R antibody, the intensity of PLA2R staining, and intensity of C3 staining, respectively.

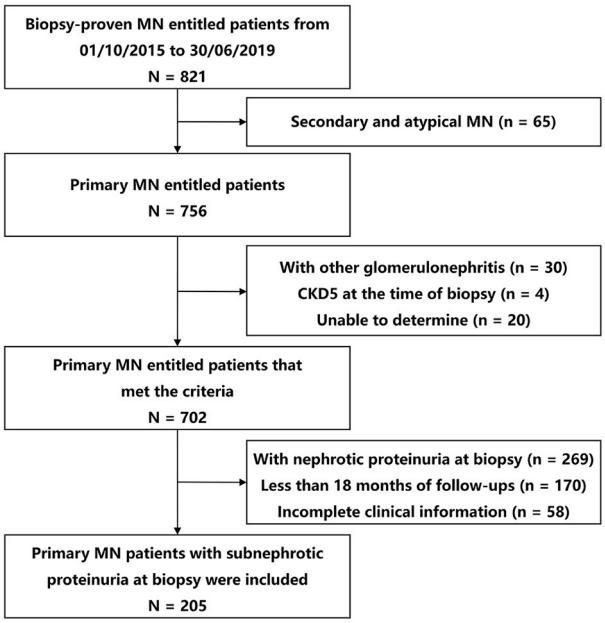

FIGURE 1 | Study flow diagram of patient selection. MN, membranous nephropathy; CKD, chronic kidney disease.

The median follow-up time was 43 months (IQR, 34–53 months). The medians of initial proteinuria and microhematuria were 1.26 g/d (IQR, 0.76–1.81 g/d) and 3.00 RBCs/HPF (IQR, 2.00–5.20 RBCs/HPF), respectively. A total of 179 (87.32%) patients received treatment with either ACEIs or ARBs. In total, 125 (60.98%) patients received statin class medications. Additionally, 132 (64.39%) patients were treated with corticosteroids and/or other IS agents during follow-up.

The numbers and sequences of the eligible patients that reached various outcomes are presented in **Figure 2**. During our observational period, 97.56% of the entire cohort (200 of 205) achieved PR. The median time from kidney biopsy to PR was 5 months (IQR, 3–8 months). Among patients that attained PR, 83.5% (167 of 200) achieved CR in a median of 12 months (IQR, 8–21 months) after kidney biopsy. To sum up, 25.85% (53 of 205)

TABLE 1 | Clinical characteristics according to the tertiles of the initial proteinuria.

Variable	Total	Initial proteinuria			*P*-value
		T1 (<0.98 g/d)	T2 (0.98–1.56 g/d)	T3 (>1.56 g/d)	
Patient No.	205	68	69	68	
Baseline					
Age (IQR), years	48 (31–55)	44 (28–55)	49 (33–54)	49 (34.5–57.5)	0.185
Females (*n*, %)	102 (49.76)	43 (63.24)	30 (43.48)	29 (42.65)	0.017
BMI, Mean ± SD, kg/m^2 190	24.12 ± 3.82	23.52 ± 3.88	24.19 ± 3.54	24.70 ± 3.96	0.052
Systolic BP, Mean ± SD, mmHg	121.40 ± 16.80	119.16 ± 19.40	120.74 ± 14.31	124.32 ± 16.16	0.052
Diastolic BP, Mean ± SD, mmHg	73.88 ± 12.00	73.01 ± 12.70	73.16 ± 11.95	75.47 ± 11.33	0.171
Hypertension (*n*, %)	64 (31.22)	20 (29.41)	20 (28.99)	24 (35.29)	0.460
Diabetes (*n*, %)	15 (7.32)	2 (2.94)	5 (7.25)	8 (11.76)	0.049
Smokers (*n*, %)	38 (18.54)	13 (19.12)	16 (23.19)	9 (13.24)	0.379
Serum creatinine, Mean ± SD, mg/dl	0.85 ± 0.16	0.83 ± 0.18	0.89 ± 0.15	0.83 ± 0.15	0.756
EGFR, Mean ± SD, ml/min/1.73 m^2	96.47 ± 19.08	96.82 ± 17.59	94.05 ± 19.71	98.57 ± 19.84	0.551
Serum albumin, Mean ± SD, g/dl	3.22 ± 0.73	3.41 ± 0.71	3.31 ± 0.75	2.94 ± 0.64	<0.001
Proteinuria (IQR), g/d	1.62 (0.90–2.22)	0.88 (0.42–1.31)	1.62 (1.05–2.30)	2.09 (1.69–2.78)	<0.001
Microhematuria (IQR), RBCs/HPF	3 (1–8)	3 (2–9)	3 (1–8)	3 (1–6)	0.167
High level of serum anti-PLA2R antibody (*n*, %) 179	73 (40.78)	20 (32.79)	23 (37.70)	30 (52.63)	0.030
High intensity of IF-PLA2R staining (*n*, %) 167	110 (65.87)	35 (62.50)	41 (70.69)	34 (64.15)	0.844
High intensity of IF-C3 staining (*n*, %) 174	24 (13.79)	6 (10.17)	9 (15.00)	9 (16.36)	0.336
Follow-up					
Initial proteinuria (IQR), g/d	1.26 (0.76–1.81)	0.64 (0.52–0.75)	1.26 (1.13–1.36)	2.16 (1.81–2.77)	<0.001
Initial microhematuria (IQR), RBCs/HPF	3.00 (2.00–5.20)	2.73 (2.00–6.40)	3.20 (2.00–4.50)	3.07 (1.50–6.05)	0.962
Follow-up duration (IQR), months	43 (34–53)	38.5 (29–49)	46 (38–54)	42.5 (35.5–53)	0.142
Treatment with ACEI/ARB (*n*, %)	179 (87.32)	60 (88.24)	61 (88.41)	58 (58.29)	0.607
Treatment with statin (*n*, %)	125 (60.98)	33 (48.53)	41 (59.42)	51 (75.00)	0.002
Treatment with corticosteroids (*n*, %)	132 (64.39)	37 (54.41)	49 (71.01)	46 (67.65)	0.108
Treatment with tacrolimus (*n*, %)	93 (45.37)	30 (44.12)	39 (56.52)	24 (35.29)	0.303
Treatment with cyclophosphamide (*n*, %)	56 (27.32)	10 (14.71)	18 (26.09)	28 (41.18)	0.001
Outcomes (*n*, %)					
Partial remission	200 (97.56)	68 (100)	68 (98.55)	64 (94.12)	0.027
Complete remission	167 (81.46)	64 (94.12)	55 (79.71)	48 (70.59)	<0.001
Nephrotic proteinuria progression	53 (25.85)	7 (10.29)	15 (21.74)	31 (45.59)	<0.001
40% decline in the eGFR	6 (2.93)	2 (2.94)	1 (1.45)	3 (4.41)	0.612
50% decline in the eGFR	1 (0.49)	0	0	1 (1.47)	0.220
End-stage renal disease	1 (0.49)	0	0	1 (1.47)	0.220

SD, standard deviation; IQR, interquartile range; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; RBCs, red blood cell counts; HPF, high-power field; PLA2R, phospholipase A2 receptor; IF, immunofuorescence; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blockers; MN, membranous nephropathy.

of the entire cohort suffered nephrotic proteinuria progression in a median of 38 months (IQR, 22–49 months), including 36 patients who suffered nephrotic proteinuria progression after reaching PR and CR, 14 after PR, and three without remission. Additionally, only 2.93% (six of 205) patients suffered ≥40% loss of kidney function, and 0.49% (1 of 205) progressed to reach the pre-defined kidney function progression endpoint.

The eligible patients were classified according to the tertiles of initial proteinuria (0.96 and 1.53 g/d) (**Table 1**). From T1 to T3, those with higher levels of initial proteinuria were more likely to be females, Diabetes, and received statin or cyclophosphamide therapy. As initial proteinuria increased, the level of serum albumin decreased, whereas the level of serum anti-PLA2R antibody increased. As for the outcomes, from T1 to T3, the proportions of PR and CR decreased, whereas the proportion of nephrotic proteinuria progression increased ($P < 0.05$).

Partial Remission

In our cohort, the 1-year and 3-year cumulative probabilities of PR were 92.68 and 98.54%, respectively. As shown in **Figure 3A**, from T1 to T3 groups, the 1-year cumulative probabilities of PR were 97.06, 97.10 and 83.82%, respectively ($P < 0.001$). The results of Cox hazards regression analyses are summarized in **Table 2** and **Supplementary Table 1**. In the multivariate model, the initial proteinuria was an independent prognostic factor of PR with a HR of 0.67 (95% CI, 0.56–0.86; $P < 0.001$).

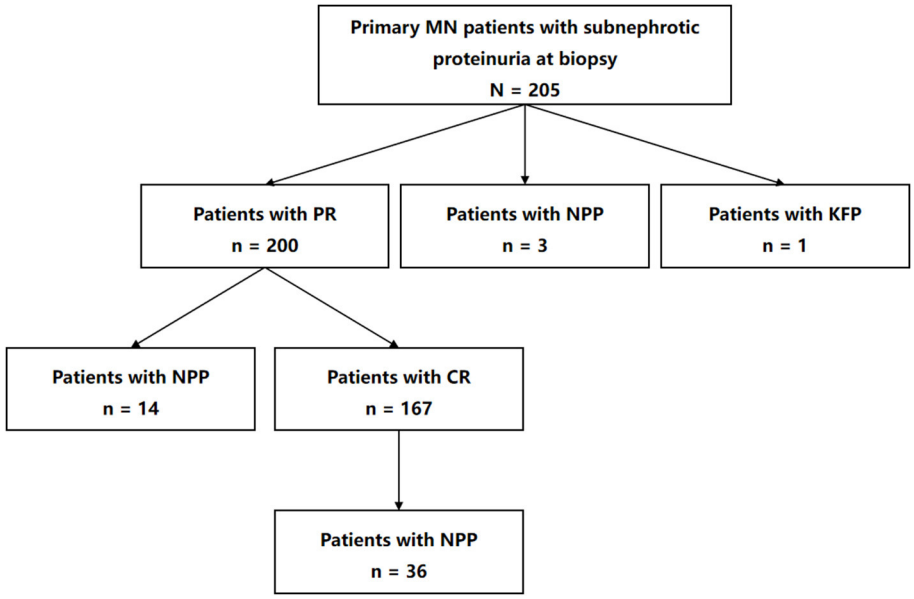

FIGURE 2 | Flow diagram of numbers and sequences of the participants that reached various outcomes in the study. MN, membranous nephropathy; PR, partial remission; NPP, nephrotic proteinuria progression; KFP, kidney function progression; CR, complete remission.

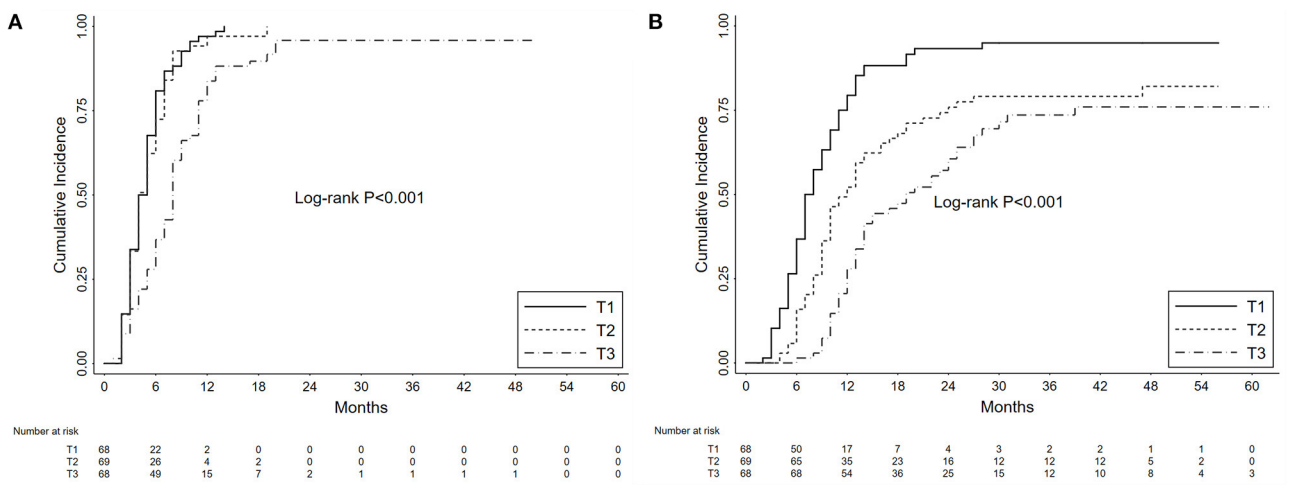

FIGURE 3 | Kaplan-Meier curves depict the cumulative probabilities of partial remission **(A)** and complete remission **(B)** for patients with primary membranous nephropathy and subnephrotic range proteinuria. The patients were grouped by the tertiles (T1 vs. T2 vs. T3) of initial proteinuria. The time zero was a kidney biopsy. Log-rank tests were used for the comparison between groups.

TABLE 2 | Univariate and multivariate analyses of independent prognostic factors of partial remission.

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Serum albumin (per 1 g/dl increase)	1.41 (1.17–1.70)	<0.001	1.20 (0.99–1.46)	0.063
Initial proteinuria (per 1 g/d increase)	0.64 (0.54–0.76)	<0.001	0.67 (0.56–0.80)	<0.001

CI, confidence interval.

TABLE 3 | Univariate and multivariate analyses of independent prognostic factors of complete remission.

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Age (per 1 year increase)	0.99 (0.98–1.00)	0.037	0.99 (0.98–1.00)	0.194
Initial proteinuria (per 1 g/d increase)	0.56 (0.45–0.70)	<0.001	0.50 (0.40–0.63)	<0.001
Treatment with corticosteroids (yes vs. no)	1.57 (1.13–2.18)	0.008	2.02 (1.43–2.85)	<0.001

CI, confidence interval.

TABLE 4 | Clinical characteristics between never nephrotic and nephrotic proteinuria progression groups.

Variable	Never nephrotic	Nephrotic progression	*P*-value
Patients No.	152	53	
Age (IQR), years	49 (33–55)	45 (27–54)	0.144
Females, *n* (%)	82 (53.95)	20 (37.74)	0.042
BMI, Mean±SD, kg/m^2	23.92 ± 3.68	24.74 ± 4.18	0.197
Hypertension, *n* (%)	51 (33.55)	13 (24.53)	0.222
Diabetes, *n* (%)	14 (9.21)	1 (1.89)	0.122
Smokers, *n* (%)	28 (18.42)	10 (18.87)	0.943
eGFR, Mean ± SD, ml/min/1.73 m^2	95.20 ± 18.39	100.12 ± 20.67	0.106
Serum albumin, Mean ± SD, g/dl	3.29 ± 0.72	3.01 ± 0.73	0.013
Initial proteinuria (IQR), g/d	1.16 (0.67–1.52)	1.78 (1.21–2.59)	<0.001
Initial microhematuria (IQR), RBCs/HPF	2.67 (1.64–4.59)	4.20 (3.00–7.75)	<0.001
High level of serum anti-PLA2R antibody, *n* (%)	51 (38.35)	22 (47.83)	0.259
High intensity of IF-PLA2R staining, *n* (%)	81 (65.85)	29 (65.91)	0.995
High intensity of IF-C3 staining, *n* (%)	17 (12.98)	7 (16.28)	0.586
ACEI/ARB, *n* (%)	134 (88.16)	45 (84.91)	0.540
Statin, *n* (%)	89 (58.55)	36 (67.92)	0.228
Corticosteroids, *n* (%)	97 (63.82)	35 (66.04)	0.771
Tacrolimus, *n* (%)	64 (42.11)	29 (54.72)	0.112
Cyclophosphamide, *n* (%)	42 (27.63)	14 (26.42)	0.864

SD, standard deviation; IQR, interquartile range; BMI, body mass index; BP, blood pressure; eGFR, estimated glomerular filtration rate; RBCs, red blood cell counts; HPF, high-power field; PLA2R, phospholipase A2 receptor; IF, immunofuorescence; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blockers.

Complete Remission

During the follow-up, the 1-year and 3-year cumulative probabilities of CR were 53.17 and 82.41%, respectively. As shown in **Figure 3B**, from T1 to T3 groups, the 1-year cumulative probabilities of CR were 79.41, 52.17, and 27.94%, respectively, and the 3-year cumulative probabilities were 94.96, 79.10, and 73.57%, respectively ($P < 0.001$). The results of Cox hazards regression analyses are presented in **Table 3** and **Supplementary Table 2**. In the multivariate model, the initial proteinuria and treatment with corticosteroids were independent prognostic factors of CR with HRs of 0.50 (95% CI, 0.40–0.63; $P < 0.001$) and 2.02 (95% CI, 1.43–2.85; $P < 0.001$), respectively.

Nephrotic Proteinuria Progression

Clinical characteristics of patients in the never nephrotic and nephrotic proteinuria progression groups are summarized in **Table 4**. Compared with patients in the never nephrotic group, those in the nephrotic proteinuria progression group showed higher levels of initial proteinuria [1.78 (IQR, 1.21–2.59) vs. 1.16 (IQR, 0.67–1.52) g/d; $P < 0.001$] and initial microhematuria [4.20 (IQR, 3.00–7.75) vs. 2.67 (IQR, 1.64–4.59) RBCs/HPF; $P < 0.001$], and a lower level of serum albumin [3.01 (SD, 0.73) vs. 3.29 (SD, 0.72) g/dl; $P = 0.013$].

The cumulative probabilities of nephrotic proteinuria progression were 13.17, 22.96, and 33.15% after 1, 3, and 5 years, respectively. As shown in **Figure 4A**, the 3-year cumulative probabilities of nephrotic proteinuria progression were 10.22, 16.65, and 41.59%, respectively, and the 5-year cumulative probabilities were 17.70, 29.00, and 51.75%, respectively, in the T1, T2, and T3 groups ($P < 0.001$). In **Figure 4B**, the 3-year and 5-year cumulative probabilities of nephrotic proteinuria progression were 16.53 and 26.67%, respectively, in the no persistent microhematuria group, and 40.27 and 51.00%, respectively, in the initial persistent microhematuria group ($P = 0.002$).

The results of Cox hazards regression analyses are summarized in **Table 5** and **Supplementary Table 3**. In the multivariate model, the female gender, initial proteinuria, and

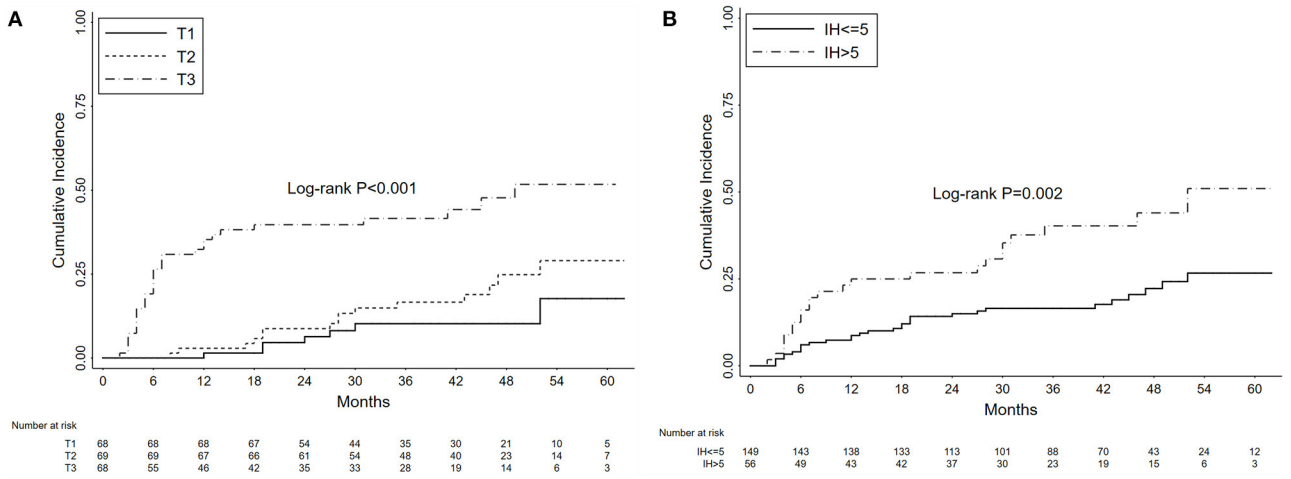

FIGURE 4 | Kaplan-Meier curves depicting the cumulative probabilities of nephrotic proteinuria progression for patients with primary membranous nephropathy and subnephrotic range proteinuria. The patients were grouped by the tertiles of initial proteinuria (T1 vs. T2 vs. T3) **(A)** and the levels of initial hematuria (IH, >5 vs. ≤5 RBCs/HPF) **(B)**. The time zero was kidney biopsy. Log-rank tests were used for the comparison between groups.

TABLE 5 | Univariate and multivariate analyses of independent prognostic factors of nephrotic proteinuria progression.

Factor	Univariate analysis		Multivariate analysis	
	Hazard ratio (95% CI)	*P*-value	Hazard ratio (95% CI)	*P*-value
Female gender (vs. male gender)	0.54 (0.31–0.94)	0.029	0.49 (0.28–0.87)	0.016
Serum albumin (per 1 g/dl increase)	0.62 (0.43–0.90)	0.011	0.84 (0.57–1.25)	0.392
Initial proteinuria (per 1 g/d increase)	2.93 (2.22–3.88)	<0.001	2.97 (2.23–3.97)	<0.001
Initial microhematuria (per 1 RBC/HPF increase)	1.08 (1.03–1.13)	0.001	1.11 (1.05–1.17)	<0.001

CI, confidence interval; RBC, red blood cell count; HPF, high-power field.

initial microhematuria were identified as independent prognostic factors of nephrotic proteinuria progression. The corresponding adjusted HRs were 0.49 (95%CI, 0.28–0.87; $P = 0.016$), 2.97 (95%CI, 2.23–3.97; $P < 0.001$), and 1.11 (95%CI, 1.05–1.17; $P < 0.001$).

Kidney Function Progression

During the course, 2.93% (six of 205) patients suffered a 40% loss of kidney function. Their main clinical characteristics are summarized in **Table 6**. Four patients (**Table 6**, patients 1, 3, 4, 6) showed the initial proteinuria values of >1.00 g/d, and four patients (**Table 6**, patients 1, 3, 4, 5) showed the initial microhematuria values of >5.00 RBCs/HPF. During follow-up, five patients (**Table 6**, patients 1, 2, 4, 5, 6) were treated with either ACEIs or ARBs, and 3 (**Table 6**, patients 3, 4, 5) were treated with IS agents. Final proteinuria >1.00 g/d was observed in three patient (**Table 6**, patients 3, 4, 6). In the end, four patients (**Table 6**, patients 1, 2, 4, 5) reached PR, and two patients (**Table 6**, patients 1, 4) suffered nephrotic proteinuria progression.

Kidney function progression was observed in only one patient (**Table 6**, patient 6). She was a 59-year-old woman whose renal biopsy showed stage II in histological classification. Baseline renal function and blood pressure were normal. The initial proteinuria and microhematuria were 2.07 g/d and 3.00 RBCs/HPF, respectively. Despite the administration of BP medications, the levels of 24 h urine protein excretion were consistently higher than 2.00 g/d during follow-up. A total of 18 months after kidney biopsy, kidney function showed an irreversible decline to a serum creatinine value of 3.43 mg/dl (eGFR, 13.63 ml/min/1.73 m^2).

DISCUSSION

Our results suggested that, among patients with primary MN and subnephrotic proteinuria at kidney biopsy, the initial proteinuria, defined as the mean proteinuria of the first 6 months during follow-up, was an independent prognostic factor of PR, CR, and nephrotic proteinuria progression. Moreover, a higher level of the initial microhematuria, defined as the mean microhematuria of the first 6 months during follow-up, was associated with an increased risk of nephrotic proteinuria progression. For all we know, this is the first study to evaluate the prognostic relevance of microhematuria in this type of patient.

As one of the most common causes of adult-onset primary nephrotic syndrome, primary MN is most typically depicted as being accompanied by nephrotic range proteinuria (1, 2, 5, 7).

TABLE 6 | Clinical characteristics of patients with 40% loss in the kidney function.

Variable	Patient 1	Patient 2	Patient 3	Patient 4	Patient 5	Patient 6
Age (years)	68	50	61	67	55	59
Gender	Female	Female	Male	Male	Female	Female
Baseline serum albumin (g/dl)	3.62	3.83	3.24	2.49	3.16	2.11
Baseline eGFR (ml/min/1.73 m^2)	69.4	80.1	92.1	78	94.0	90.0
Initial proteinuria (g/d)	1.62	0.23	2.50	1.37	0.79	2.07
Initial microhematuria (RBCs/HPF)	5.5	2.4	5.2	11	9.5	3
Final serum albumin (g/dl)	3.72	4.52	3.51	3.71	4.24	2.32
Final eGFR (ml/min/1.73 m^2)	35.5	50.4	51	39.1	52.6	13.6
Final proteinuria (g/24 h)	0.81	0.25	2.05	7.48	0.13	2.25
ACEI/ARB treatment	Yes	Yes	No	Yes	Yes	Yes
Immunosuppressive treatment	No	No	CS+TAC	CS+CTX	CS	No
Follow-up (months)	36	43	50	52	54	18

EGFR, estimated glomerular filtration rate; RBCs, red blood cell counts; HPF, high-power field; ACEI, angiotensin converting enzyme inhibitor; ARB, angiotensin receptor blockers; CS, corticosteroids; TAC, tacrolimus; CTX, cyclophosphamide.

For those in the subnephrotic status, guidelines classified most of them as a low-risk group and recommended conservative treatment (22, 23). However, close follow-up of these patients remains important, as a significant proportion of the patients who present with low-level of proteinuria will evolve to nephrotic range proteinuria and then follow a course similar to the classic nephrotic at-presentation group (7). Overall, our results showed that the prognosis of this subset of patients was generally excellent. During a median follow-up of 43 months, 200 (97.56%), 167 (81.46%), and 53 (25.85%) patients reached PR, CR, and nephrotic proteinuria progression, respectively. The 1-year, 3-year, and 5-year cumulative incidences of nephrotic proteinuria progression were 13.17, 22.96, and 33.15%, respectively. Only one patient (0.49%) progressed to the kidney function progression, defined as $\geq$50% loss of kidney function or ESRD.

One of the primary findings of our study was that the initial proteinuria, referred to as the average proteinuria of the first 6 months during the course, was an independent predictor of PR, CR, or nephrotic proteinuria progression. In the multivariate analyses, the corresponding aHRs were 0.67 (95% CI, 0.56–0.80), 0.50 (95% CI, 0.40–0.63), and 2.97 (95% CI, 2.23–3.97), respectively. The result suggested that the dynamic detection and effective management of proteinuria contributed to improving the prognosis of this subset of patients. In the cohort of Hladunewich et al. the only distinguishing baseline feature between the never nephrotic group and the nephrotic post-presentation group was a higher level of proteinuria in the group that subsequently developed nephrotic syndrome [1.98 (IQR, 0.3–3.4) vs. 2.43 (IQR, 0.5–3.4) g/d]. Compared with the proteinuria at baseline, the advantage of initial proteinuria was that it not only avoided the bias and instability of single-point detection but also depicted the initial treatment response of proteinuria. It is reasonable to expect more large-scale and prospective studies to confirm the clinical implications of our findings.

Microscopic hematuria has been previously considered as an indicator for a future flare in patients with systematic lupus erythematosus (SLE). Ding et al. suggested that changes in urinary sediments, either isolated microscopic hematuria or accompanied by sterile pyuria, were related to the disease activity among patients with SLE (13). Rhee et al. suggested that cumulative duration of microscopic hematuria was a possible biomarker of subsequent nephritis relapse in ANCA-associated vasculitis (11). In our study, among patients with primary MN and subneprotic proteinuria at kidney biopsy, the initial microhematuria was an independent risk factor of nephrotic proteinuria progression, and the corresponding aHR was 1.11 (95% CI, 1.05–1.17) in the multivariate analysis. The result highlighted the prognostic value of microhematuria for the nephrotic proteinuria progression in those with primary MN and low-grade proteinuria. Though the underlying mechanism is unclear, multiple studies have suggested that persistent glomerular microhematuria might represent a continued "low-grade" activity of the underlying inflammatory process, which could stimulate kidney injury through the oxidative stress caused by the release of hemoglobin and iron from broken RBCs into renal tubular cells (9, 17, 18, 24–27). Additionally, more studies are needed to verify our findings and elucidate the prognostic relevance of microhematuria among this subset of patients. It could contribute to more reasonable monitoring and guidance of clinical medication use in this group of primary MN patients.

Our study also had several limitations. First, it was a single-center retrospective cohort accomplished with a review of medical records. The interpretation might be biased owing to selection error. Second, we did not make regression analyses because the number of patients who developed kidney function progression was relatively small. Even if we did, an excessively small number of outcome events could increase the risk of an unstable conclusion. So the influence of some confounding factors on the results could not be excluded. Furthermore, there was no standardized regimen for induction and maintenance therapy, and the

treatment decisions were totally dependent on the preference of individual physicians. Therefore, these fundamental restrictions could not be avoided in the evaluation of the effect of each treatment. Additionally, the tests for serum anti-PLA2R antibody titers were conducted using the IIFAs, which enable only semi-quantitative measurement of the serum anti-PLA2R antibody. For follow-up research and monitoring, enzyme-linked immunosorbent assay (ELISA) is more suitable. However, before august 2017, the ELISA was not done in our nephrology laboratory.

In summary, our data indicated that primary MN patients presenting with subnephrotic proteinuria overall had a benign prognosis. During our follow-up period, ∼80% of patients achieved complete remission of proteinuria, and only ∼20% of patients re-developed nephrotic range proteinuria. The initial proteinuria was an independent predictor of PR, CR, or nephrotic proteinuria progression. Besides, the initial microhematuria might be an additional indicator for the nephrotic proteinuria progression and provide reference indices for clinicians to monitor and manage this subset of primary MN patients.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Xijing Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LH, PH, and HW designed the study, analyzed the data, and drafted the manuscript. PH, JL, and YZ collected and entered data. LH and HW contributed to data acquisition and interpretation. All authors read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.737700/full#supplementary-material

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Microhematuria Enhances the Risks of Relapse and Renal Progression in Primary Membranous Nephropathy

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Objective: To determine whether there is an association between microhematuria and relapse or kidney disease progression in patients with primary membranous nephropathy (PMN).

Methods: A cohort of 639 patients with biopsy-proven PMN from two centers was followed for a median of 40 months. The exposures were initial hematuria, time-averaged hematuria, and cumulative duration of hematuria. The outcomes were relapse and renal progression, which were defined by a 40% reduction in renal function or end-stage renal disease. Cox proportional hazards regression and competing risk analyses were performed to yield hazard ratios (HRs) and subdistribution hazard ratios (sHRs) with 95% confidence intervals (CIs). Sensitivity and interaction analyses were also performed.

Results: After adjusting for confounders, a higher level of initial hematuria was associated with a 1.43 (95% CI, 1.15–1.78) greater hazard of relapse. Worsening hematuria remarkably increased the risk of short-term relapse (HR, 4.64; 3.29–6.54). Time-averaged hematuria (sHR, 1.35; 1.12–1.63) and cumulative duration of hematuria (sHR, 1.17; 1.02–1.34) were independent predictors of renal progression. Hematuria remission was related to a reduced risk of renal progression over time in patients with positive microhematuria (sHR, 0.63; 0.41–0.96).

Conclusions: A higher level of initial hematuria was a remarkable predictor of relapse in patients with PMN, and the magnitude and persistence of microhematuria were independently associated with kidney disease progression.

Keywords: primary membranous nephropathy, microscopic hematuria, relapse, kidney disease progression, remission

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INTRODUCTION

Glomerulonephritis (GN) may be caused by problems with the body's immune system. Damage to the glomeruli causes blood and protein loss in the urine. Microhematuria, accompanied by variable amounts of proteinuria, is one of the most common clinical manifestations of GN, e.g., IgA nephropathy (IgAN), ANCA-associated vasculitis (AAV), and lupus nephritis (1–3). The significance of hematuria in the natural history of renal disease is not clear (4–6). Some experts regard persistent hematuria as a hallmark of ongoing disease activity, but other experts posit that

stable hematuria is a benign lesion resulting from prior kidney damage (5, 6). The role of hematuria in the development of glomerular diseases received unprecedented attention recently. Several studies showed that persistent hematuria was independently associated with kidney disease progression among patients with IgAN (7–9). Another study suggested that the presence of persistent hematuria, but not proteinuria, was a significant predictor of renal relapse in patients with AAV and kidney involvement (10).

Primary membranous nephropathy (PMN) is a kidney-specific, autoimmune glomerular disease, and it is one of the most common causes of nephrotic syndrome in non-diabetic adults worldwide (11–14). Approximately 80% of patients present with nephrotic-range proteinuria (≥3.5 g/d), and the remaining 20% have subnephrotic proteinuria (12–17). Renal function is normal at presentation in more than 90% of patients (12, 15–18). The most alarming long-term outcome of PMN is progressive loss of renal function, which occurs in ∼60% of untreated patients, with 35% eventually reaching end-stage renal disease (ESRD) within 10 years (12, 13, 16, 17, 19, 20). The long-term renal survival of patients with persistent non-nephrotic proteinuria is >80–90% at 10 years (21). Other established predictors include age, male sex, decreased estimated glomerular filtration rate (eGFR) on presentation, persistent elevation of phospholipase A_2 receptor antibody (anti-PLA_2R) levels after therapy, and C3 staining in the biopsy sample (12, 13, 15–17).

Microhematuria is not uncommon, and appears in ∼50–60% of patients with PMN (11–13, 15, 18). However, few studies systematically analyzed the prognostic relevance of microhematuria over time in PMN. The effects of microhematuria on disease activity and renal survival are not clear. In this study, we present a two-center longitudinal cohort of participants with biopsy-proven PMN. The present study (1) examined whether initial hematuria was an independent predictor for relapse of nephrotic range proteinuria and (2) investigated the magnitude and persistence of microhematuria over time in relation to kidney disease progression in patients with PMN.

METHODS

Study Population

From 1 October 2015 to 30 June 2019, patients with biopsy-proven PMN from two centers in Xi'an, China, (the nephrology departments of Xijing Hospital [XH] and Shaanxi Province Hospital of Traditional Chinese Medicine [SPHTCM]) participated in this retrospective cohort. Other inclusion criteria were (1) a baseline (established at the time of renal biopsy) eGFR >15 ml/min/1.73 m^2, calculated according to the Chronic Kidney Disease Epidemiology Collaboration equation (22), (2) at least 18 months of follow-up, and (3) sufficient information on treatments and laboratory parameters for investigation. Patients with secondary membranous nephropathy, atypical membranous nephropathy, or other concomitant glomerular diseases were excluded. The Ethics Committee of Xijing Hospital approved the study.

Data Collection

Each participant attended regular visits at intervals of 3–12 months. Urine sediment and 24-h proteinuria excretion were tested at each visit and recorded prospectively. Skilled clinical examiners performed microscopic analyses of urine sediments. Each urine sample was strictly analyzed within 2 h of collection and reported as red blood cells (RBCs)/high-power field (HPF). We only recorded the results of urine sendiments, mainly of glomerular derived RBCs (dysmorphic RBCs > 70%). Since our study was focused on the microhematuria, the results of microscopic analyses during gross hematuria episodes were excluded. Other clinical data, including age, sex, body mass index, blood pressure, serum creatine, serum albumin, and use of renin-angiotensin-aldosterone system (RAAS) blockades and immunosuppressive (IS) agents during the first year, were systematically recorded. The follow-up data were last updated on 31 December 2020.

Definitions and Outcomes

The follow-up time referred to the interval between kidney biopsy and the last outpatient visit, death, or ESRD, whichever occurred first. ESRD was defined as eGFR<15 ml/min/1.73 m^2 or chronic dialysis. Hypertension was defined as systolic blood pressure ≥140 mmHg, diastolic blood pressure ≥90 mmHg, or the use of antihypertension drugs.

For PMN, nephrotic syndrome referred to proteinuria ≥3.5 g/d and serum albumin <3 g/dL. Complete remission (CR) was defined as proteinuria <0.3 g/d with serum albumin ≥3.5 g/dL and stable kidney function. A stable kidney function was defined as an eGFR that remained unchanged or declined by <40%. Partial remission (PR) was proteinuria >0.3 but <3.5 g/d plus a ≥50% reduction from baseline level, with serum albumin ≥3.5 g/dL and stable kidney function. No remission (NR) was defined as proteinuria ≥3.5 g/d, a <50% decline in proteinuria, serum albumin <3.5 g/dL, or a ≥40% decline in the eGFR prior to achieving proteinuria reduction. Relapse was defined as a reappearance of proteinuria ≥3.5 g/d after PR or CR.

The variables of interest were initial hematuria and the magnitude and persistence of microhematuria over time. Due to the high variability of microscopic assessment at a single time point, we treated the average hematuria of the first 6 months during follow-up as the initial hematuria. An initial hematuria of >5 RBCs/HPF was defined as initial persistent hematuria. The magnitude of microhematuria was expressed using time-averaged hematuria (TA-H). According to previously reported methods (8, 9), TA-H was the mean of the average hematuria, which was calculated for every 6-month block for each person. Time-averaged proteinuria (TA-P) was calculated in the same manner. The persistence of microhematuria was expressed using the cumulative duration of hematuria (CD-H), which was the sum of the number of months with a microhematuria count >3 RBCs/HPF. Persistent hematuria was defined by TA-H >5 RBCs/HPF or CD-H >12 months.

The primary outcome was relapse. We evaluated time to first event for relapse with start date defined as the time a patient first attained PR. As an exploratory study, the association between worsening hematuria and short-term (within 12 months) relapse

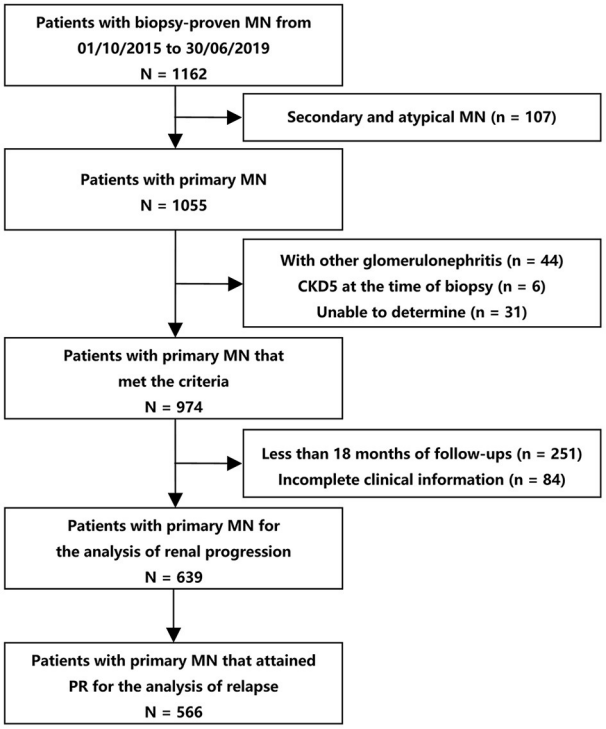

FIGURE 1 | Flow diagram of patient selection. The patients that reached PR ($N = 566$) were used for the analyses of the association between initial hematuria and relapse. All the eligible patients ($N = 639$) were included for the analyses of the association between microhematuria and kidney disease progression. CKD, chronic kidney disease; MN, membranous nephropathy; PR, partial remission.

was also analyzed. The worsening hematuria was defined as the appearance of positive hematuria (>3 RBCs/HPF). In this part, the exposures were the appearance of positive hematuria within 12 months before relapse (vs. no appearance) and the specific time frames (concurrent with relapse, over the past 3 months, over the past 4 to 6 months, or over the past 7 to 12 months) that the positive hematuria appeared in.

The secondary outcome was renal progression, which was a composite endpoint that included a ≥40% decline in eGFR or ESRD. The time zero was kidney biopsy. For futher research, the relationship of hematuria remission with renal progression was also quantified. Hematuria remission was defined by the absence of microhematuria or the presence of ≤3 RBCs/HPF in all urine sediment tests performed during at least 12 months before the last outpatient visit. More details on the methods are provided in the supplementary material (**Supplementary Appendix 1**, **Supplementary Table 1**).

Statistical Analysis

Continuous variables are presented as means with standard deviations (SD) for normal distributions or medians with interquartile range (IQR) for skewed distributions. Categorical variables are presented as percentages. Differences between groups were compared using Student's *t*-tests for normally distributed variables, Kolmogorov-Smirnov tests for variables with skewed distributions, and chi-squared tests for categorical variables. The cumulative probability of relapse was estimated using the Kaplan-Meier method and compared using the log-rank test. Adjusted analyses were performed using Cox proportional hazards regression. The proportionality assumption of models was evaluated *via* the examination of Shoenfeld residuals. For renal progression, the outcome probability was evaluated using univariate analyses with cumulative incidence function (CIF) methods and Gray test and multivariate competing risk regression analyses. Death without renal progression was treated as a competing event. TA-H, CD-H, and TA-P were included as time-varying covariates. Initial hematuria, TA-H, CD-H, and TA-P were log-transformed due to their positively skewed distribution. To avoid data loss due to transformation, 0.1 was added to the values of initial hematuria, TA-H, and CD-H. The strength of association is expressed as a hazard ratio (HR) or subdistribution hazard ratio (sHR) with 95% confidence intervals (95% CIs).

Sensitivity analyses were performed by (1) restricting the subcohort to a single center or (2) using a 50% decline in renal function or ESRD as the endpoint. We also tested whether nephrotic syndrome (vs. subnephrotic status) and the use of RAAS blockers or IS agents modified the effect of initial hematuria or hematuria classification by adding the interaction terms in the models. A two-sided P value <0.05 was considered significant. Statistical analyses were performed using Stata Edition 15.0 (StataCorp., College Station, TX, USA).

RESULTS

Patients and Outcomes

Among 1,162 participants from the two centers, 639 were eligible for analyses (474 from XH and 165 from SPHTCM) (**Figure 1**). At baseline, data were missing for 4.69% (30/639) and 0.78% (5/639) of patients for body mass index and microhematuria, respectively. Participant clinical characteristics are described in **Table 1**. There were 409 (64.01%) males, and the median age was 49 (IQR, 37–59) years. A total of 236 (36.93%) patients had hypertension, and 311 (48.67%) presented with nephrotic syndrome. The mean eGFR was 95.92 ± 20.54 mL/min/1.73 m^2, and the serum albumin was 2.88 ± 0.76 g/dL. The median proteinuria was 3.33 (1.75–5.10) g/d, and the microhematuria was 3 (1–8) RBCs/HPF at baseline.

The median follow-up was 40 (28–49) months. The median initial hematuria was 3.2 (2–6.23) RBCs/HPF. The median TA-H was 2.34 (1.28–3.78) RBCs/HPF, CD-H was 3 (1–8) months, and TA-P was 1.19 (0.65–2.07) g/d. During follow-up, 566 (88.58%) patients in the entire cohort achieved PR. Among patients who reached PR, 111 (19.61%) subsequently relapsed in a median of 30 (18–41) months. Overall, 50 (7.82%) patients reached renal progression, including 9 ESRD events.

Microhematuria and Relapse

The patients who attained PR (566 patients) were used for the analyses of this part (**Figure 1** and **Supplementary Table 1**). The exposure was the initial hematuria. **Figure 2** shows the

TABLE 1 | Characteristics of patients with primary membranous nephropathy.

Variable	Total	TA-H		*P* value	CD-H		*P* value
		>5 RBCs/HPF	≤5 RBCs/HPF		>12 months	≤12 months	
No. of patients	639	93	546		95	544	
Baseline							
Age (yr)	49 (37–59)	49 (38–58)	49 (37–59)	0.961	53 (41–62)	49 (36–58)	0.053
Males, *n* (%)	409 (64.01)	70 (75.27)	339 (62.09)	0.014	68 (71.58)	341 (62.68)	0.096
BMI (kg/m^2)	24.6 (22.2–26.9)	24.6 (22.7–26.6)	24.6 (22.2–27.0)	0.747	24.6 (22.3–27.3)	24.5 (22.2–26.8)	0.910
Hypertension, *n* (%)	236 (36.93)	31 (33.33)	205 (37.55)	0.437	36 (37.89)	200 (36.76)	0.833
Nephrotic syndrome, *n* (%)	311 (48.67)	53 (56.99)	258 (47.25)	0.082	48 (50.53)	263 (48.35)	0.695
Serum creatine, mg/dL	0.87 ± 0.23	0.87 ± 0.20	0.87 ± 0.23	0.728	0.88 ± 0.26	0.86 ± 0.22	0.403
eGFR (ml/min per 1.73 m^2)	95.92 ± 20.54	96.69 ± 20.80	95.79 ± 20.51	0.695	94.35 ± 22.44	96.19 ± 20.20	0.419
Serum albumin (g/dL)	2.88 ± 0.76	2.59 ± 0.75	2.93 ± 0.75	0.001	2.73 ± 0.73	2.90 ± 0.76	0.035
Microhematuria (RBCs/HPF)	3 (1–8)	7 (3–19)	3 (1–7)	<0.001	6 (3–18)	3 (1–7)	<0.001
Proteinuria (g/d)	3.33 (1.75–5.1)	3.76 (2.13–6.4)	3.18 (1.7–4.9)	0.196	3.51 (1.83–5.11)	3.29 (1.72–5.06)	0.675
Follow-up							
Follow-up duration (months)	40 (28–49)	31 (24–47)	41 (29–50)	0.001	42 (27–50)	40 (28–49)	0.591
Initial hematuria (RBCs/HPF)	3.20 (2.00–6.23)	8.57 (3.67–13.75)	2.98 (1.67–5.00)	<0.001	7.00 (3.20–11.00)	3.00 (1.73–5.08)	<0.001
TA-H (RBCs/HPF)	2.34 (1.28–3.78)	7.43 (5.98–8.83)	2.1 (1.14–2.94)	<0.001	6.00 (4.27–8.55)	2.10 (1.14–2.99)	<0.001
CD-H (months)	3 (1–8)	15 (9–23)	2 (1–6)	<0.001	19 (16–25)	2 (1–5)	<0.001
TA-P (g/d)	1.19 (0.65–2.07)	1.85 (1.19–3.05)	1.09 (0.61–1.91)	<0.001	2.10 (1.32–3.44)	1.09 (0.61–1.83)	<0.001
RAAS blockades, *n* (%)	496 (77.62)	63 (67.74)	433 (79.30)	0.013	67 (70.53)	429 (78.86)	0.072
IS agents				0.059			0.015
Monotherapy, *n* (%)	93 (14.55)	20 (21.51)	73 (13.37)		23 (24.21)	70 (12.87)	
Combination therapy, *n* (%)	434 (67.92)	62 (66.67)	372 (68.13)		58 (61.05)	376 (69.12)	
Outcome							
No remission, *n* (%)	73 (11.42)	33 (35.48)	40 (7.33)	<0.001	35 (36.84)	38 (6.99)	<0.001
Partial remission, *n* (%)	566 (88.56)	60 (64.52)	506 (92.67)	<0.001	60 (63.16)	506 (93.01)	<0.001
Relapse, *n* (%)	111 (19.61)	33 (55.00)	78 (15.42)	<0.001	33 (55.00)	78 (15.42)	<0.001
Complete remission, *n* (%)	385 (68.02)	34 (56.67)	351 (69.78)	0.046	33 (55.00)	352 (69.57)	0.022
Renal progression[a], *n* (%)	50 (7.82)	14 (15.05)	36 (6.59)	0.007	18 (18.95)	32 (5.88)	<0.001
ESRD, *n* (%)	9 (1.41)	4 (4.30)	5 (0.92)	0.010	7 (7.37)	2 (0.37)	<0.001

BMI, body mass index; CD-H, cumulative duration of hematuria; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HPF, high-power field; IS agents, immunosuppressive agents; RAAS, renin-angiotensin-aldosterone system; RBCs, red blood cell counts; TA-H, time-average hematuria; TA-P, time-average proteinuria.
[a] Renal progression was defined as a 40% decline in the eGFR or ESRD.

Kaplan-Meier curves for relapse in PMN patients with and without initial persistent hematuria. The 3-year and 5-year cumulative probabilities of relapse were 28.36 and 31.81%, respectively, in patients with initial persistent hematuria, and 17.46 and 27.31%, respectively, in patients without initial persistent hematuria ($P = 0.036$).

The results of Cox proportional hazards regression analyses are presented in **Table 2** and **Supplementary Table 2**. A higher level of initial hematuria was an independent risk factor for relapse (adjusted HR, 1.43; 95% CI, 1.15–1.78) in Model C after adjustment for age, sex, hypertension, serum albumin, eGFR, proteinuria, and use of RAAS blockers and IS agents. As a categorical variable, initial persistent hematuria was associated with a 1.52 (1.02–2.29) greater hazard of relapse in reference to patients without initial persistent hematuria.

The effect size was recalculated in a single center in sensitivity analyses. The adjusted HR of initial hematuria in the XH center (**Supplementary Table 3**) was 1.49 (1.15–1.94) in Model C. The corresponding adjusted HR was 1.57 (1.00–2.48) in the SPHTCM center (**Supplementary Table 4**). Between the nephrotic and subnephrotic groups, no heterogeneity was observed for the association of initial hematuria with relapse (P for interaction $= 0.471$). Neither the use of RAAS blockers (P for interaction $= 0.397$) nor IS agents (P for interaction $= 0.850$) significantly modified the effect of initial hematuria.

Worsening Hematuria and Short-Term Relapse

In this part, the patients that reached PR (566 patients) were used for the analyses (**Figure 1** and **Supplementary Table 1**). As shown in **Table 3** and **Supplementary Table 5**, the appearance of positive hematuria substantially enhanced the risk of short-term relapse, in reference to no appearance, after adjustment for negative conversion of hematuria and other confounders (adjusted HR, 4.64; 3.29–6.54). Compared to a flare concurrent with hematuria, worsening hematuria was associated with a

higher risk of relapse within the next 4–6 months (adjusted HR, 1.57; 1.04–2.38).

Microhematuria and Renal Progression

All eligible participants (639 patients) were included in the analysis of the association of microhematuria with renal progression (**Figure 1** and **Supplementary Table 1**). The exposures were the initial hematuria, TA-H, and CD-H. According to the magnitude of microhematuria over time, 15.05% (14/93) and 6.59% (36/546) of patients with PMN reached composite renal progression events, respectively, in the persistent hematuria (TA-H >5 RBCs/HPF) and non-persistent hematuria (TA-H ≤5 RBCs/HPF) groups. As for the persistence of microhematuria, 18.95% (18/95) and 5.88% (32/544) of patients reached composite renal progression events, respectively, in the persistent hematuria (CD-H >12 months) and non-persistent hematuria (CD-H ≤12 months) groups (**Table 1**).

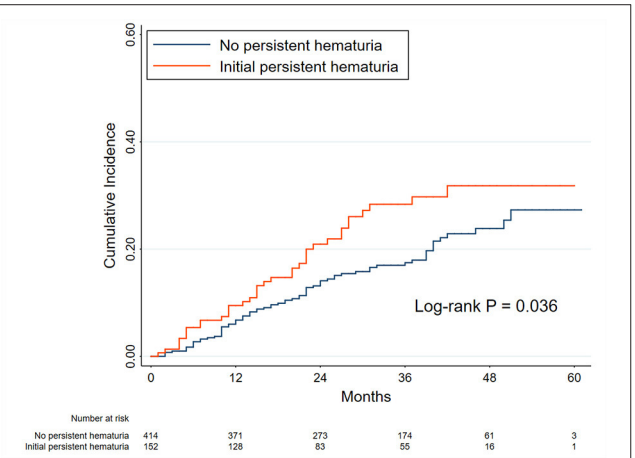

FIGURE 2 | Kaplan-Meier curves for relapse in patients with and without initial persistent hematuria. The initial hematuria was defined as the average hematuria of the first 6 months of follow-up in patients with primary membranous nephropathy. The initial persistent hematuria was defined as an initial hematuria >5 RBCs/HPF. Between-group comparison was performed using the log-rank test. The time zero was when a person first attained partial remission.

As shown in **Table 4** and **Supplementary Table 6**, initial hematuria might not be associated with renal progression (adjusted sHR, 1.25; 0.93–1.67), but TA-H (adjusted sHR, 1.35; 1.12–1.63) and CD-H (adjusted sHR, 1.17; 1.02–1.34) were independent predictors of renal progression after adjustment for age, sex, hypertension, serum albumin, eGFR, proteinuria, and use of RAAS blockers and IS agents in the competing risk regression Model C. According to the magnitude of microhematuria over time, persistent hematuria was associated with a greater risk of renal progression ($P = 0.040$, **Figure 3A**). As for the persistence of microhematuria, similar result was observed ($P = 0.019$, **Figure 3B**).

The clinical characteristics of the patients grouped by centers are presented in **Supplementary Table 7**. The results of sensitivity analyses from each center were consistent with the entire cohort. The adjusted sHRs in the XH center (**Supplementary Table 8**) were 1.55 (1.21–1.98) and 1.27 (1.09–1.48) for TA-H and CD-H, respectively. The corresponding adjusted sHRs in the SPHTCM center (**Supplementary Table 9**) were 1.12 (0.82–1.54) and 1.04 (0.90–1.20), respectively. The results were consistent when the endpoint was a 50% decline in renal function or ESRD, and the corresponding adjusted sHRs were 1.38 (1.12–1.71) and 1.23 (1.00–1.51), respectively, for TA-H and CD-H (**Figures 3C,D** and **Supplementary Table 10**). Interaction analyses also showed no significant heterogeneity of the association between TA-H and renal progression (nephrotic syndrome, P for interaction = 0.868; use of RAAS blockers, P for interaction = 0.824; use of IS agents, P for interaction = 0.405). Similarly, no significant heterogeneity was observed in the analyses of the association between CD-H and renal progression (nephrotic syndrome, P for interaction = 0.869; use of RAAS blockers, P for interaction = 0.495; use of IS agents, P for interaction = 0.288).

Hematuria Remission and Renal Progression

Patients with positive hematuria (TA-H >3 RBCs/HPF, 220 patients) were used for the analyses, and were divided into hematuria remission (71 patients) and non-remission (149 patients) groups (**Supplementary Table 1**). The exposure was the hematuria remission, which was clearly defined in the methods. Five (7.04%) patients in the remission group and

TABLE 2 | Microhematuria and risk of relapse in cox proportional hazards models.

Factor	Hazard ratio for relapse (95% CI); *P* value			
	Unadjusted	Model A	Model B	Model C
Initial hematuria (per 1 unit greater)	1.44 (1.17–1.79); 0.001	1.46 (1.18–1.81);<0.001	1.44 (1.16–1.79); 0.001	1.43 (1.15–1.78);0.001
Initial persistent hematuria (in reference to no persistent hematuria)	1.52 (1.02–2.25); 0.038	1.58 (1.06–2.35);0.024	1.54 (1.03–2.29); 0.036	1.52 (1.02–2.29);0.042

The initial hematuria, defined as the average hematuria of the first 6 months, was log-transformed. To avoid data loss, 0.1 was added to the value of initial hematuria. The initial persistent hematuria, defined as an initial hematuria value >5 RBCs/HPF, was expressed as a binary variable. Model A was adjusted for age, sex, and hypertension; sex and hypertension were expressed as categorical variables. Model B was adjusted for covariates in Model A plus baseline serum albumin, eGFR, and proteinuria. Model C was adjusted for covariates in Model B plus use of RAAS blockers and IS agents. Use of RAAS blockers and IS agents were expressed as categorical variables.

CI, confidence interval; eGFR, estimated glomerular filtration rate; IS agents, immunosuppressive agents; RAAS, renin-angiotensin-aldosterone system.

TABLE 3 | Worsening hematuria and risk of short-term relapse in cox proportional hazards models.

Factor	Hazard ratio for relapse (95% CI); *P* value			
	Unadjusted	**Model A**	**Model B**	**Model C**
Appearance of positive hematuria (vs. no appearance)	3.83 (2.76–5.33); <0.001	4.66 (3.31–6.56);<0.001	4.66 (3.31–6.55); <0.001	4.64 (3.29–6.54);<0.001
Time of hematuria appearance				
Concurrent with relapse	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)	1.00 (Reference)
Over past 3 mo	0.92 (0.65–1.29); 0.627	1.14 (0.80–1.62);0.466	1.10 (0.77–1.57); 0.589	1.12 (0.78–1.59);0.536
Over past 4 to 6 mo	0.91 (0.66–1.25); 0.560	1.48 (0.98–2.21);0.060	1.51 (1.00–2.26); 0.048	1.57 (1.04–2.38);0.031
Over past 7 to 12 mo	0.76 (0.58–0.98); 0.034	1.15 (0.81–1.63);0.426	1.12 (0.79–1.59); 0.534	1.13 (0.80–1.61);0.492

Worsening hematuria and negative conversion of hematuria were included as time-varying covariates. Model A was adjusted for negative conversion of hematuria, age, sex, and hypertension; negative conversion of hematuria, sex and hypertension were expressed as categorical variables. Model B was adjusted for covariates in Model A plus baseline serum albumin, baseline eGFR, and proteinuria. Model C was adjusted for covariates in Model B plus RAAS blockers and IS agents. Use of RAAS blockers and IS agents were expressed as categorical variables.

CI, confidence interval; eGFR, estimated glomerular filtration rate; IS agents, immunosuppressive agents; RAAS, renin-angiotensin-aldosterone system; TA-P, time-averaged proteinuria.

TABLE 4 | Microhematuria and risk of renal progression in competing risk regression models.

Factor	Subdistribution hazard ratio for renal progression (95% CI); *P* value			
	Unadjusted	**Model A**	**Model B**	**Model C**
Initial hematuria (per 1 unit greater)	1.37 (1.08–1.74); 0.009	1.38 (1.08–1.76);0.009	1.26 (0.95–1.67); 0.104	1.25 (0.93–1.67);0.137
Initial persistent hematuria (in reference to no persistent hematuria)	2.33 (1.34–4.05); 0.003	2.43 (1.38–4.27);0.002	2.19 (1.19–4.05); 0.012	2.19 (1.17–4.12);0.015
TA-H (per 1 unit greater)	1.45 (1.23–1.69); <0.001	1.47 (1.25–1.74);<0.001	1.34 (1.11–1.61); 0.002	1.35 (1.12–1.63);0.002
Persistent hematuria based on TA-H (in reference to no persistent hematuria)	2.73 (1.47–5.05); 0.001	2.87 (1.55–5.32);0.001	2.07 (1.07–4.01); 0.030	2.01 (1.03–3.92);0.040
CD-H (per 1 unit greater)	1.25 (1.09–1.44); 0.001	1.24 (1.09–1.41);0.001	1.17 (1.03–1.34); 0.020	1.17 (1.02–1.34);0.021
Persistent hematuria based on CD-H (in reference to no persistent hematuria)	3.38 (1.91–5.99); <0.001	3.12 (1.75–5.55);<0.001	2.24 (1.15–4.35); 0.018	2.22 (1.14–4.33);0.019

Renal progression was defined as a 40% decline in the eGFR or ESRD. Death without renal progression was treated as a competing event. TA-H, CD-H, and TA-P were included as time-varying covariates. Initial hematuria, TA-H, CD-H, and TA-P were log-transformed due to their positively skewed distribution. To avoid data loss due to transformation, 0.1 was added to the values of initial hematuria, TA-H, and CD-H. Initial persistent hematuria was defined by initial hematuria >5 RBCs/HPF. Persistent hematuria was defined by TA-H >3 RBCs/HPF or CD-H >12 months. The initial persistent hematuria and persistent hematuria were expressed as binary variables. Model A was adjusted for age, sex, and hypertension; sex and hypertension were expressed as categorical variables. Model B was adjusted for covariates in Model A plus baseline serum albumin, baseline eGFR, and TA-P. Model C was adjusted for covariates in Model B plus use of RAAS blockers and IS agents. Use of RAAS blockers and IS agents were expressed as categorical variables.

CD-H, cumulative duration of hematuria; CI, confidence interval; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; IS agents, immunosuppressive agents; RAAS, renin-angiotensin-aldosterone system; TA-H, time-averaged hematuria; TA-P, time-averaged proteinuria.

28 (18.79%) patients in the non-remission group reached composite renal progression events (**Supplementary Table 11**). Hematuria remission, included as a time-varying covariate, was substantially associated with a reduced risk of renal progression in Model C (adjusted sHR, 0.63; 0.41–0.96) (**Table 5** and **Supplementary Table 12**). CIF analysis revealed that the incidence of renal progression considerably decreased in the remission group compared to the non-remission group ($P = 0.034$, **Figure 4**).

DISCUSSION

Taken together, the results from our double-center cohort suggested that (1) the initial hematuria was an independent risk factor for relapse, and the magnitude and persistence of microhematuria over time were substantially associated with kidney disease progression in patients with PMN; (2) the worsening of microhematuria markedly enhanced the hazard of short-term relapse; and (3) the remission of microhematuria exhibited a positive effect on the improvement in renal outcomes in patients with PMN and microhematuria during follow-up.

Hematuria is a clinical symptom of some renal disorders with benign features, and it has been neglected by many clinicians for decades. However, multiple recent studies challenged this concept and demonstrated that the presence of hematuria, especially of glomerular origin, was a hallmark of inflammation and pathological damage in nephridial tissue (4–6, 23). The accumulation of heavy microhematuria over time may be a predictor of adverse outcomes, e.g., disease activity

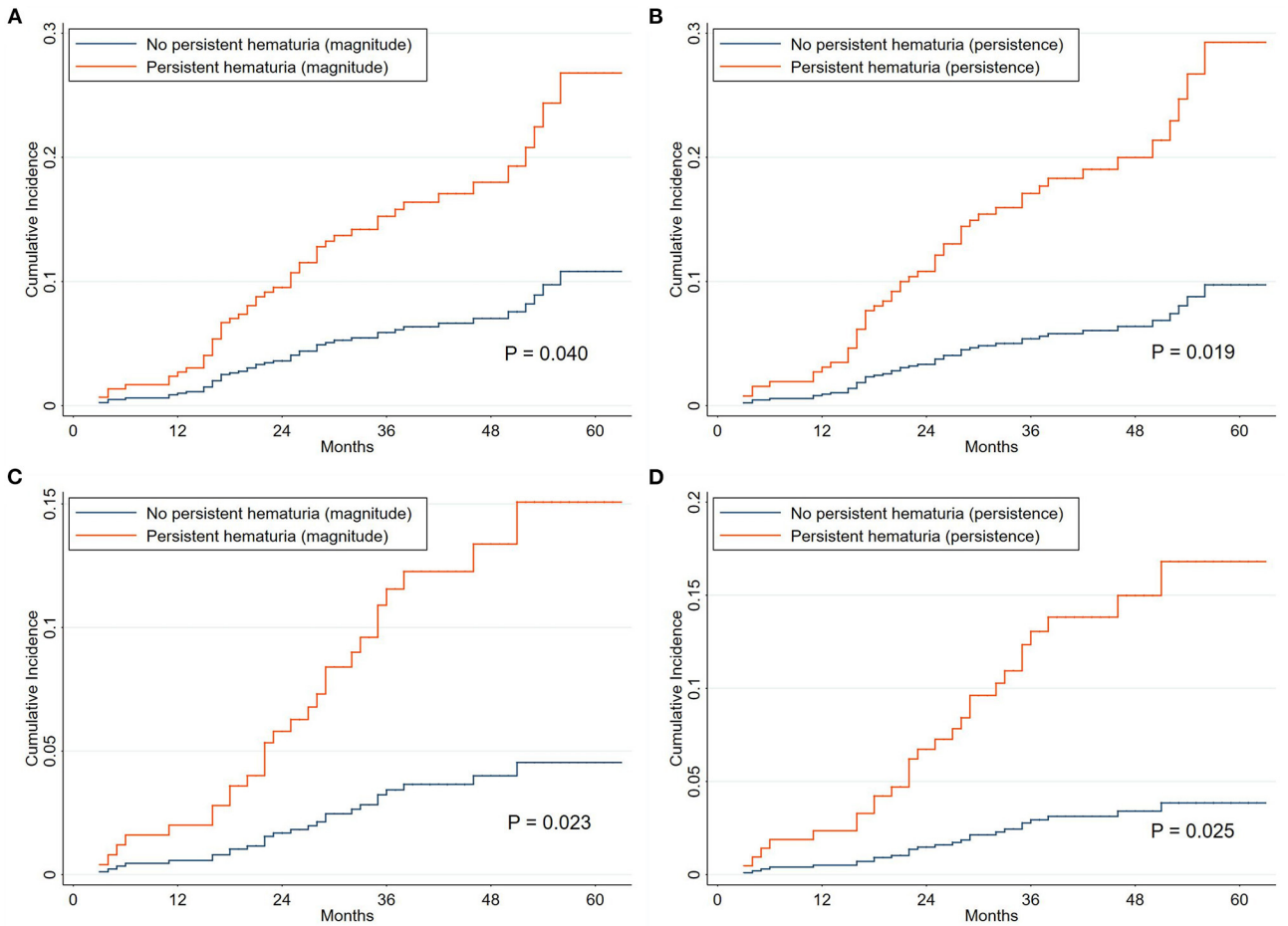

FIGURE 3 | Cumulative incidence curves for composite renal progression events grouped by hematuria classification. The cumulative incidence function methods and Gray test were used for analyses. The time zero was kidney biopsy. According to the magnitude of microhematuria over time, the persistent hematuria was defined as time-averaged hematuria >5 RBCs/HPF **(A,C)**. As for the persistence of microhematuria, the persistent hematuria was defined as cumulative duration of hematuria >12 months **(B,D)**. The corresponding endpoints were 40% decline in renal function or end-stage renal disease **(A,B)** and 50% decline in renal function or end-stage renal disease **(C,D)**. Death without renal progression was treated as a competing event.

TABLE 5 | Hematuria remission and risk of renal progression.

Factor	Sub-distribution hazard ratio for renal progression (95% CI); *P* value			
	Unadjusted	**Model A**	**Model B**	**Model C**
Hematuria remission	0.61 (0.40–0.93); 0.023	0.63 (0.41–0.96); 0.033	0.64 (0.41–0.98); 0.040	0.63 (0.41–0.96); 0.034

Renal progression was defined as a 40% decline in eGFR or ESRD. Death without renal progression was treated as a competing event. Hematuria remission, included as a time-varing covariate, was the absence of hematuria or the presence of ≤3 RBCs/HPF in all the urine sediment tests performed during at least 12 months before the last outpatient visit. Model A was adjusted for age, sex, and hypertension; sex and hypertension were expressed as categorical variables. Model B was adjusted for covariates in Model A plus baseline serum albumin, baseline eGFR, and TA-P; TA-P was log-transformed and included as a time-varying covariate. Model C was adjusted for covariates in Model B plus RAAS blockades and IS agents. Use of RAAS blockades and IS agents were expressed as categorical variables.

CI, confidence interval; eGFR, estimated glomerular filtration rate; ESRD, end-stage renal disease; HPF, high power field; IS agents, immunosuppressive agents; RAAS, renin-angiotensin-aldosterone system; TA-P, time-averaged proteinuria.

in systemic lupus erythematosus (24, 25), renal relapse in AAV (10, 26, 27), and kidney disease progression in IgAN (7–9).

The most prominent clinical feature of PMN is nephrotic syndrome and its associated manifestations. Microhematuria is not uncommon and accounts for ∼50% of patients at disease onset and 60% during the course (11–13, 15, 18, 28, 29). However, few studies focused on the prognostic relevance of the magnitude and persistence of microhematuria over time in PMNs. A total of 316 (49.45%) patients in our double-center cohort had

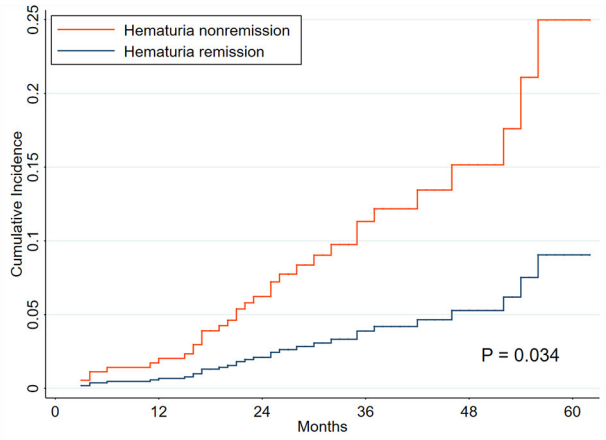

FIGURE 4 | Cumulative incidence curves for composite renal progression events grouped by hematuria remission. The cumulative incidence function methods and Gray test were used for analyses. The time zero was kidney biopsy. The endpoint was renal progression, defined as a 40% decline in renal function or end-stage renal disease. Death without renal progression was treated as a competing event.

urinary erythrocyte counts of >3 RBCs/HPF at baseline, and 220 (34.43%) patients had positive hematuria (TA-H >3 RBCs/HPF) during follow-up.

Previous studies generally used TA-H >5 RBCs/HPF to represent persistent hematuria (8, 9). However, 37 patients in our cohort had TA-H values >5 RBCs/HPF but a CD-H <12 months, and 8 patients had a CD-H <6 months. Obviously, the application of the previous definition in these patients would not objectively reflect the real status of hematuria during the disease course. Our study quantified the magnitude and persistence of microhematuria over time using TA-H and CD-H, respectively.

Our results suggested that the initial persistent hematuria was independently associated with a greater risk of a future flare in patients with PMN. One of the greatest future challenges is the establishment of a clear association between glomerular damage and the potential utility of initial persistent hematuria to decide when it is necessary to increase IS treatment. Moreover, there was an obvious connection between worsening hematuria and the risk of a flare within the following 12 months. The appearance of positive hematuria was related to an increased risk of relapse in reference to no appearance. Notably, the worsening of hematuria over the past 4–6 months presented a more prominent warning compared to the occurrence of hematuria at the onset of a relapse. These findings support the routine use of urine sediments in clinical practice and emphasize the significance of close clinical monitoring in patients with PMN because this may be an early predictor of short-term relapse.

The hazards of kidney disease progression in PMN patients with persistent hematuria was significantly higher than those with non-persistent hematuria during followup. It has been widely accepted that glomerular hematuria resulted from the passage of erythrocytes from the glomerular capillary into the urinary space and was associated with glomerular filtration barrier damage. In primary glomerulonephritis or autoimmune diseases, infiltrating leukocytes may release metalloproteinases and reactive oxygen species, leading to a glomerular basement membrane which is more susceptible to rupture (5, 30). Futhermore, persistent glomerular hematuria might represent a continued "low-grade" activity of the underlying inflammatory process. This could also induce renal damage *via* the oxidative stress caused by the release of hemoglobin and iron from broken erythrocytes into renal tubular cells (5, 8, 30–34). On the other hand, the remission of hematuria independently reduced the hazard of renal progression by 37% in patients with positive microhematuria. The search for adequate surrogate markers of kidney disease progression is a key issue in many clinical conditions (5, 16). Therefore, we suggest that hematuria remission may be a surrogate marker of renal survival for patients with PMN and microhematuria. However, the relationship between hematuria remission and hard endpoints must be confirmed in prospective and long-term cohorts.

Our study has the following strengths. (1) This was the first study to investigate the prognostic relevance of microhematuria over time in PMN. (2) The magnitude and persistence of microhematuria over time were systematically quatified. (3) The relationships between the worsening hematuria and short-term relapse, and between hematuria remission and kidney disease progression were also analyzed. (4) The two-center design made it possible to validate the robustness of our findings.

Our study is not without its limitations. (1) This study was a retrospective cohort with a single ethnicity. (2) We did not include comprehensive data that may be associated with renal survival in patients with PMN, such as serum anti-PLA_2R levels after therapy, urinary excretion of β^2 microglobulin, and other known or unknown confounders. Therefore, we cannot exclude the possibility of residual confounding. (3) The relatively low number of renal progression events may affect the accuracy of the results.

In summary, our study showed that initial hematuria was an independent predictor for relapse, and the magnitude and persistence of microhematuria over time were independently linked with a higher risk of renal function loss in patients with PMN. Further studies are needed to verify our findings and clarify the role of surveillance and treatment of hematuria in clinical management decisions for PMN.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Xijing Hospital. Written informed consent from the participants' legal guardian/next of kin was not required to participate in this

study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

LH, PH, SS, CH, and HW: designed the study, analyzed the data, and drafted the manuscript. PH, JL, and YZ: collected and entered data. LH, PH, XY, SS, CH, and HW: contributed to data acquisition and interpretation. All authors have read and approved the final manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.704830/full#supplementary-material

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CASE REPORT
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Case Report: Membranous Nephropathy Secondary to Cobalamin C Disease

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Background: Mutation of *MMACHC* causes inherited cobalamin C disease with methylmalonic academia (MMA) and homocysteinemia. Renal complications may also be present in patients with this deficiency. However, membranous nephropathy secondary to cobalamin C disease has not been reported to date.

Case Presentation: We encountered a 17-year-old female patient with a trans-compound mutation of *MMACHC* who presented with membranous nephropathy, MMA, homocysteinemia, and hyperuricemia. The mutations of c.80A>G (chr1:45966084) and c.482G>A (chr1:45974520) (predicting p.Gln27Arg and p.Arg161Gln missense changes at the amino acid level) had been inherited from her father and mother, respectively. Hydroxocobalamin, betaine, and L-carnitine were administered. The patient achieved complete remission of the membranous nephropathy and resolution of the MMA, homocysteinemia, and hyperuricemia.

Conclusion: Membranous nephropathy secondary to cobalamin C disease is reversible with timely intervention.

Keywords: secondary membranous nephropathy, cobalamin C disease, MMACHC, methylmalonic academia, hydroxylcobalamin

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INTRODUCTION

Many glomerular diseases are complicated by poor renal outcomes, especially some inherited diseases such as Alport syndrome. However, certain types of glomerular injury secondary to some inherited diseases may be reversible. Mutation of *MMACHC* causes cobalamin C disease (cblC), which is the most common genetic defect of cobalamin metabolism. The downstream intracellular synthesis of adenosylcobalamin and methylcobalamin, coenzymes for the enzymes methylmalonyl-coenzyme A mutase and methionine synthase, are thus disturbed, causing elevated methylmalonic acid and homocysteine with decreased methionine production. This disorder results in heterogeneous clinical presentations, both early-onset and late-onset, in patients of a wide range of ages. The main features are growth retardation, poor feeding and lethargy, hemolytic uremic syndrome, chronic thrombotic microangiopathy, developmental delay, and progressive encephalopathy and leukoencephalopathy (1).

Renal complications associated with cblC are uncommon and often do not represent the initial presentation, making them more likely to be ignored. Of these complications, thrombotic microangiopathy, chronic tubulointerstitial nephritis, and renal tubular acidosis are commonly

reported (2). However, related glomerular diseases are infrequent; only one case of focal segmental glomerulosclerosis and one case of membranoproliferative glomerulonephritis have been reported to date (3, 4). Membranous nephropathy (MN) associated with cblC has not been identified. We herein report a proband who presented with MN secondary to trans-compound mutations of *MMACHC* and was successfully treated with vitamin B replacement therapy.

CASE PRESENTATION

A 17-year-old girl presented to our nephrology department with a 7-month history of intermittent lower extremity edema, proteinuria, and hematuria. An initial renal biopsy performed at another hospital 4 months before presentation to our center indicated possible IgA nephropathy, and she was therefore prescribed monotherapy with the angiotensin receptor blocker valsartan. However, her clinical presentation was refractory to this treatment. She reported no history of drug use, infections, or malignancy and had no family history of hepatitis B or C, HIV, rheumatic disease, or tumors. She was normotensive, and a general physical examination and funduscopic examination were unremarkable. Her lung fields were clear with no rales or fremitus. A 24-h urine protein test revealed a total protein level of 2.75 g. Urinalysis revealed 24 erythrocytes per high-power field. Her serum concentrations of urea, creatinine, and albumin were within normal limits. Autoantibody test results were unremarkable. Serum antiphospholipase A2 receptor antibodies were negative. The serological results are shown in **Supplementary Table 1**. Renal ultrasound findings were normal.

The histopathological analysis of the previous renal biopsy specimen was revisited. The biopsy revealed 22 glomeruli, 1 (4.5%) of which showed global sclerosis and 1 (4.5%) of which showed focal segmental sclerosis. Mild mesangial expansion, glomerular basement membrane thickening, endothelial swelling, focally swollen podocytes, and hypercellularity were observed (**Figure 1**). Swelling of the tubular epithelium was also noted. A patchy infiltration of lymphocytes and monocytes was present within the interstitial area. There was no interstitial fibrosis. The capillary walls of the interstitial area showed no lesions. An immunohistochemical assay showed granular deposits along the capillary walls for IgM, C3, C1q, and kappa and lambda light chains as well as mild staining for IgG and IgA. IgG subclass staining showed segmental positivity for IgG1 but negativity for IgG2, IgG3, IgG4, and antiphospholipase A2 receptor.

The granular deposits along the outside of the capillary walls noted on the immunohistochemical assay, especially for lambda and C1q, indicated immune complexes along the capillaries. Spike-like projections were noted at the 3-o'clock position under silver staining, indicating a spiked glomerular basement membrane around the immune complexes. Masson staining showed scattered fuchsinophilic deposits, which denoted immune complexes. The IgG deposits in patients with primary MN are predominantly IgG4, whereas other isotypes have been identified in certain causes of secondary MN (5, 6). In this case, the lesions of mesangial cells, endothelial cells, and podocytes were inconsistent with primary MN. Lupus nephritis was excluded because of the absence of systemic manifestations of lupus, and all immunoserological markers were negative except the antinuclear antibody titer (1:100). Neither a solid tumor nor hematological malignancy was shown by computed tomography or hematological examinations, and all tumor biomarkers were negative. These findings led to the diagnosis of secondary MN.

The peak serum homocysteine concentration was 164 μmol/L (reference range, <15 μmol/L) (**Figure 2**), and the uric acid concentration was 625.7 μmol/L (reference range, 50–410 μmol/L). The blood propionyl-L-carnitine concentration was 4.39 μmol/L (reference range, 0.35–3.36 μmol/L), the ratio of propionyl-L-carnitine to acetyl-L-carnitine was 0.38 (reference range, 0.02–0.25), and the urine methylmalonic

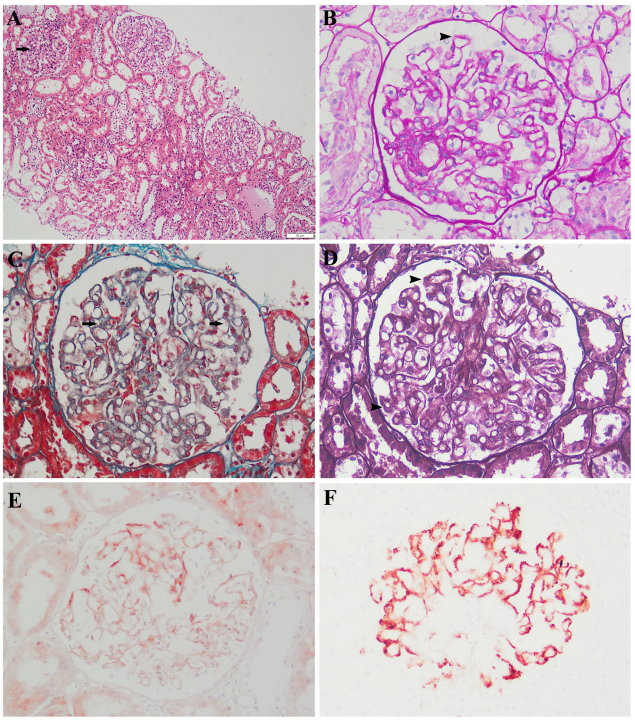

FIGURE 1 | Histologic and immunohistochemical features of renal lesions. **(A)** Segmental mesangial expansion (hematoxylin and eosin; original magnification, ×100). **(B,D)** Slight global glomerular basement membrane thickening (periodic acid–Schiff and Jones methenamine silver, respectively; original magnification, ×200). **(C)** Scattered fuchsinophilic deposits (Masson's trichrome; original magnification, ×200). Immunohistochemical assay (original magnification, ×200) showed **(E)** mild staining for IgG and **(F)** fine granular deposits of c1q.

Abbreviations: cblC, cobalamin C disease; MMA, methylmalonic academia; OHCbl, hydroxocobalamin.

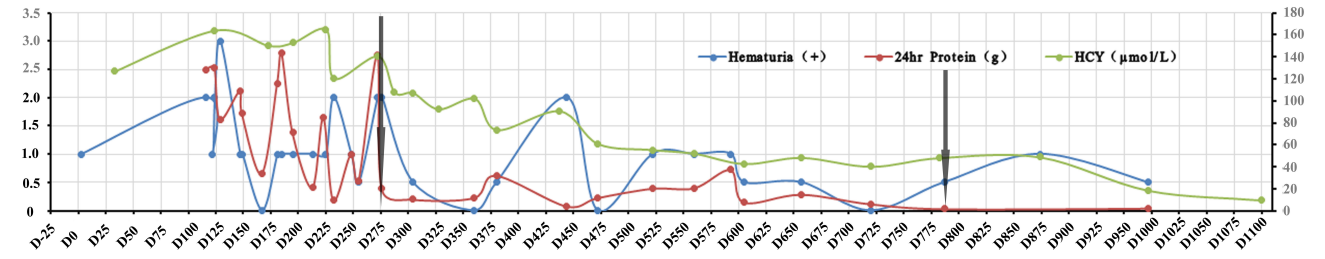

FIGURE 2 | Changes in hematuria, proteinuria, and serum homocysteine during treatment. The X-axis indicates the time points of treatment and follow up, the left Y-axis represents the 24-h urine protein and hematuria, and the right Y-axis represents the serum homocysteine concentration. The green curve denotes the serum homocysteine concentration, the red curve represents hematuria (0.5 indicates + −, 1 indicates +, 2 indicates ++, and 3 indicates + + +), and the blue curve represents the 24-h urine protein. The left arrow indicates the time of initial OHCbl treatment, and the right arrow indicated the time of revised treatment.

acid concentration was 5.1 μmol/L (reference range, 0.2–3.6 μmol/L). Genetic testing revealed compound heterozygous mutations of *MMACHC* (*NM_015506*), which confirmed late-onset cblC. The family pedigree is shown in **Figure 3A**. None of the patient's family members showed clinical symptoms. The proband was a compound heterozygote for two transitions: one in exon 1 (chr1:45966084, c.80A>G) and the other in exon 4 (chr1:45974520, c.482G>A) of *MMACHC* (**Figure 3B**). These predicted p.Gln27Arg and p.Arg161Gln missense changes at the amino acid level. The proband's father, mother, and brother were heterozygous for Q27R, R161Q, and Q27R, respectively. Therefore, the MN was considered secondary to a cobalamin-related remethylation disorder. The patient was treated with hydroxocobalamin (OHCbl) at 1 mg/week intramuscularly, betaine at 1,000 mg/day, and L-carnitine at 2,000 mg/day orally. Her serum homocysteine concentration gradually decreased, and the proteinuria and hematuria resolved during a period of over 1 year. Because of weight gain, however, the dosages were subsequently changed to OHCbl at 1 mg twice a week intramuscularly, betaine at 1,000 mg twice a day, and L-carnitine at 2,000 mg twice a day orally. At the 36-month follow-up visit, the patient's serum homocysteine concentration was 9.57 μmol/L and her serum uric acid concentration was 330.91 μmol/L (**Figure 2**). Her urinary protein concentration and erythrocyte count were within the reference ranges. The patient is being followed-up to date.

DISCUSSION

We have herein described a young female patient with secondary MN associated with compound heterozygous mutations of *MMACHC*. A high serum homocysteine concentration was noted after 4 months of treatment with an angiotensin receptor blocker. Blood and urine organic acid testing as well as genetic sequencing were performed. The pathological features of this case were consistent with secondary MN. The patient had no other relevant history. The findings in this case led to a definitive diagnosis of cblC. However, given the deposition of multiple immunoglobulins and C1q as well as the patient's age, we will continue to follow up this patient because of the possibility of lupus in the future. The patient was treated with OHCbl, betaine, and L-carnitine, effectively resolving her renal lesions, proteinuria, and hematuria. The patient's treatment outcome also supports our conclusion that the glomerular lesions were secondary to a deficiency of cobalamin metabolism.

cblC is an inborn error of cobalamin metabolism with autosomal recessive inheritance. The proband in this case was a trans-compound heterozygote with c.80A>G and c.482G>A mutations from both parents. Neither mutation has been proven to be associated with renal involvement. Neither variant is a single-nucleotide polymorphism, and both are more common in Chinese individuals (7, 8). The function of MMACHC protein is impaired in affected patients, causing methylmalonic academia (MMA) and homocysteinemia. This disorder results in diverse clinical presentations, early-onset or late-onset, in patients of a wide range of ages. Patients with c.80A>G mutation seem to show early-onset signs (8), whereas c.482G>A mutation is usually associated with a late-onset pattern (9). Our patient had a combination of c.80A>G and c.482G>A and developed late-onset signs.

MMA and homocysteinemia are common features of cblC, and the clinical manifestations can be diverse. Neurological symptoms are most often seen. Kidney injury accompanying these initial presentations in patients with early-onset disease would be more likely to be noticed by pediatric doctors (10). Diagnosis of the late-onset type is still challenging. In the present case, the diagnosis took 9 months after initiation of the renal injury. Some patients are not diagnosed until their condition has progressed to irreversible kidney injury (11). We previously described another 20-year-old female patient with IgA nephropathy associated with cblC who presented with neurological symptoms. The patient was homozygous for c.452A>G (p.His151Arg). Her serum creatinine concentration was 3.02 mg/dL by the time of diagnosis (12). It is of great importance to consider these inborn errors of cobalamin metabolism when unexplained renal injury is accompanied by neurological presentations and MMA and/or homocysteinemia.

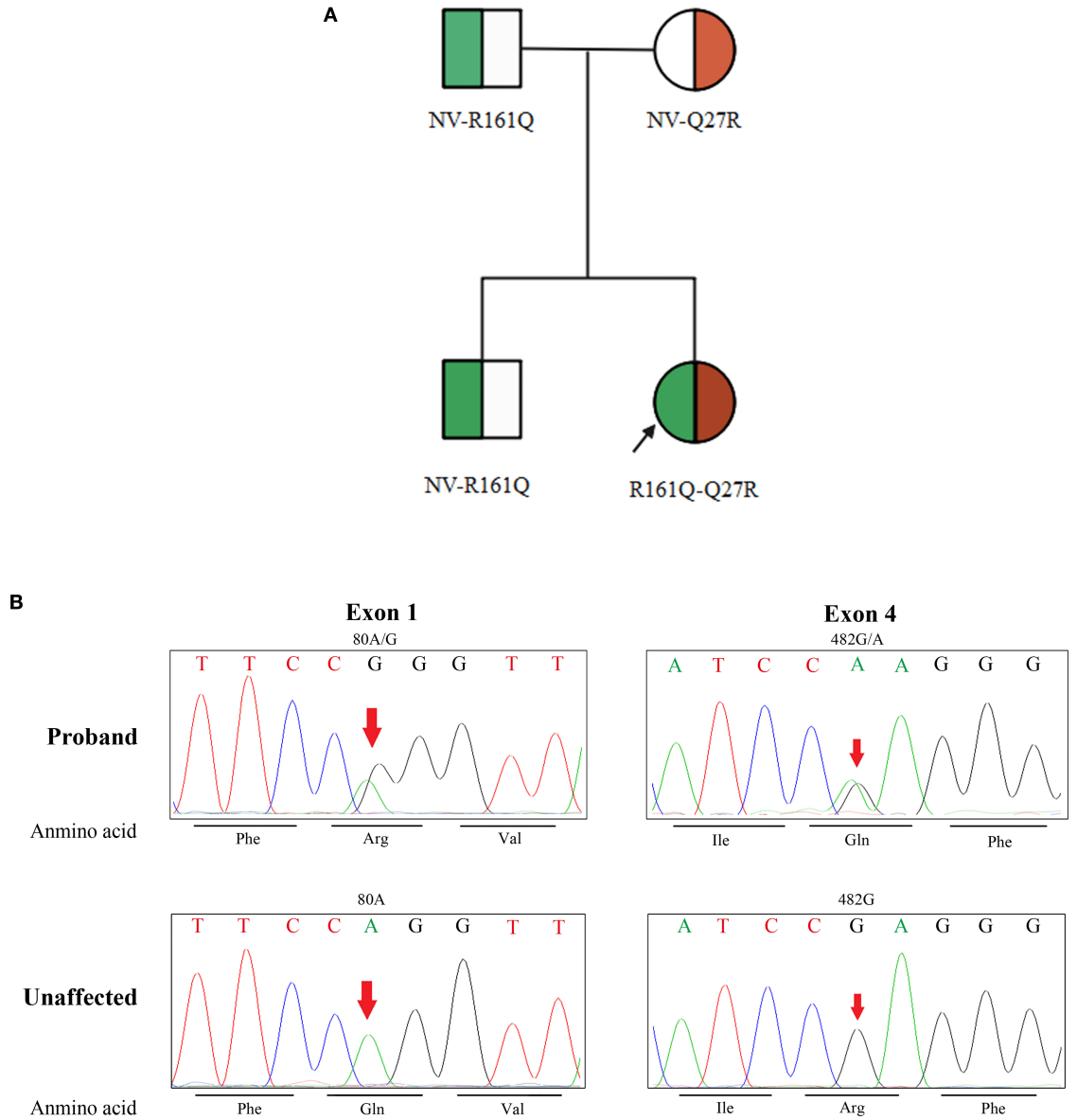

FIGURE 3 | Family pedigree and identification of *MMACHC* mutation. **(A)** Shows the family pedigree. Squares indicate male family members, and circles represent female family members. The arrow indicates the proband. The symbol of both green and red in the circle represent heterozygote. NV, non-variant. **(B)** Shows the mutated locus of the DNA sequences. Phe, phenylalanine; Arg, arginine; Val, valine; Ile, isoleucine; Gln, glutamine; Unaffected, unaffected family members.

The treatment of renal complications in patients with late-onset disease is similar to that in patients without renal involvement. The goal is to target the primary disease, normalize the serum methionine and homocysteine concentrations, and resolve the MMA. OHCbl and betaine treatment should be initiated once cblC is suspected (1). Folinic acid and levocarnitine are alternative options in some patients. Treatment by a low-protein diet in patients with cblC is controversial because the homocysteine content in dietary protein is very low (13). Thus, no diet control was applied in the present case. According to the 2021 Kidney Disease: Improving Global Outcomes guideline, complete remission is defined as a first-morning or 24-h protein/creatinine ratio of ≤200 mg/g (≤20 mg/mmol) (or negative or trace dipstick) on three or more consecutive occasions (14). Complete remission of the renal injury and normalization of cobalamin metabolism was achieved in this case. Considering the poor renal outcome of glomerular diseases, it is noteworthy that the glomerular injury secondary to cblC is reversible with timely treatment.

Interestingly, our patient's serum uric acid concentration gradually returned to the reference range as the serum

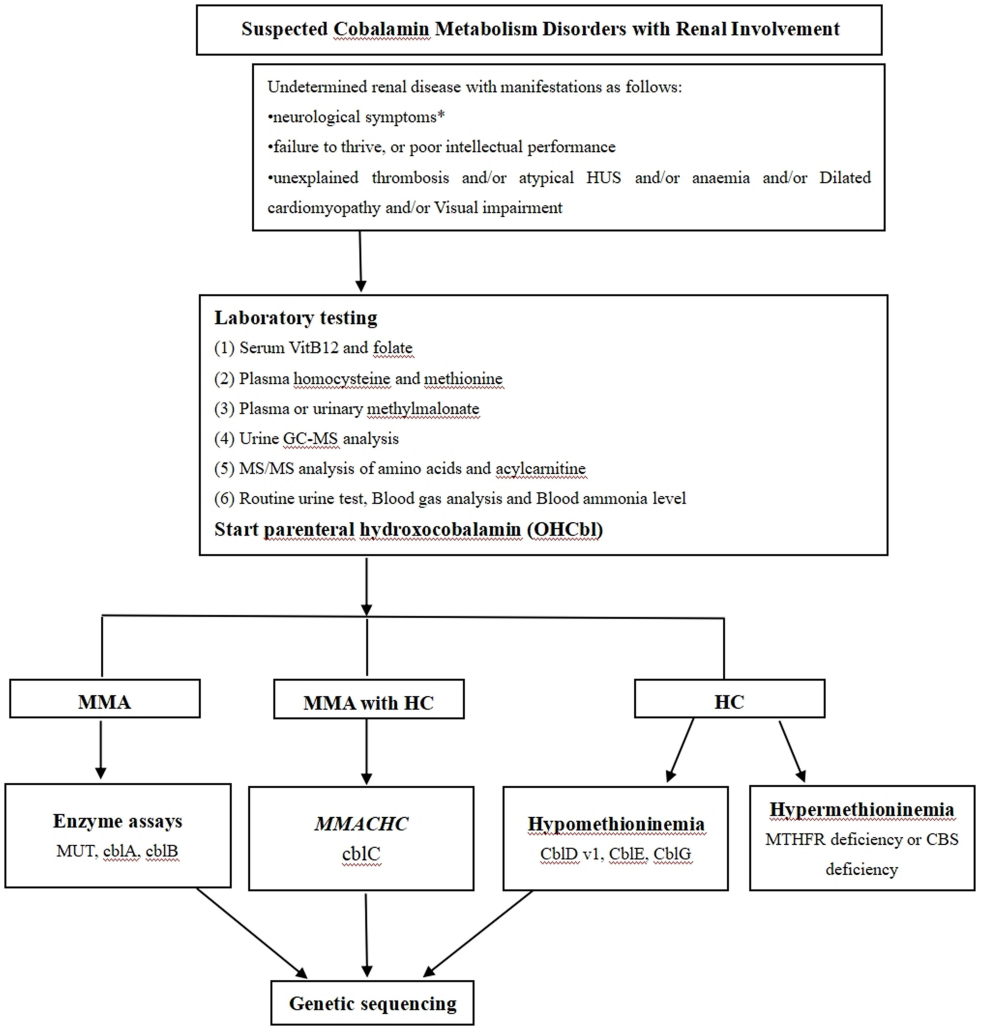

FIGURE 4 | The proposed diagnostic algorithm for suspected cobalamin metabolism disorders with renal involvement. Based on biochemical, enzymatic and genetic complementation analysis, several classes of cobalamin metabolism disorders are known as follows: mut0 /mut- type, cblA type, cblB type, cblC type, cblD-Var1 type, cblD-Var2 type, cblD type, cblF type, cblE type, cblG type, cblJ type, and cblX type; HUS: Hemolytic uremic syndrome; HC: Hyperhomocysteinaemia; MUT: Methylmalonyl-CoA mutase; MMACHC: Gene responsible for methylmalonic acidemia and homocystinuria, cblC type; MTHFR: Methylenetetrahydrofolate reductase; CBS: cystathionine-beta-synthase; * Neurological symptoms include psychiatric symptoms, cognitive impairment, ataxia neuropathy, unsteady gait, myelopathy, as well as seizures.

homocysteine concentration decreased. A possible explanation is that purine metabolism is involved in cobalamin metabolism. During the cycle in which methionine is changed to homocysteine, methionine is converted to adenosylhomocysteine (1), and the latter is split into homocysteine and adenosine (15); adenosine is then metabolized into uric acid. When this cycle is blocked by the transformation of homocysteine to methionine, adenosine accumulates, resulting in an increased level of uric acid. However, betaine facilitates the conversion of homocysteine to methionine with ensuing normal function of the cycle. A clinical correlation between homocysteinemia and hyperuricemia has also been established (16). A murine model showed that betaine can reduce the serum uric acid concentration (17). The findings of these previous studies make the above explanation more reasonable.

The high concentration of serum homocysteine was the only clue that led us to suspect cobalamin deficiency in this case. We also propose a diagnostic algorithm for suspected cobalamin deficiency with renal involvement in adult patients (1, 18–20) (**Figure 4**). This case had two limitations. First, we had no electron microscopy results because no renal tissue was left after performing slicing two times. Second, complete remission was evaluated without renal pathological evidence.

CONCLUSION

We have reported the first known case of MN secondary to cblC, which was found to be reversible with timely intervention. Further follow-up and additional cases will be necessary to clarify the pathogenesis of secondary MN.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Materials**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Qilu Hospital (Qingdao), Cheeloo College of Medicine, Shandong University. Written informed consent to participate in this study was provided by the participants' legal guardian/next of kin.

AUTHOR CONTRIBUTIONS

ZH: study design and manuscript revision. QiaW: manuscript writing. YG, QiW, and ZG: data collection. CT: figure editing. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2021.807017/full#supplementary-material

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Different Types of ANCA Determine Different Clinical Phenotypes and Outcome in ANCA-Associated Vasculitis (AAV)

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Aim: Accumulating evidence supports the use of antineutrophil cytoplasmic antibody (ANCA) type to classify different clinical entities. We aimed to evaluate whether the presence and type of ANCA determine different diseases, based on clinical phenotypes, renal involvement, and response to treatment.

Patients and Methods: Differences in terms of clinical manifestations, disease activity, laboratory parameters, and histology were recorded between patients with focal necrotizing glomerulonephritis (FNGN) due to myeloperoxidase (MPO-), proteinase 3-ANCA(+) [PR3-ANCA(+)], and ANCA(-) disease at time of diagnosis. Patients were treated with the same protocol and followed-up for 24 months, in a scheduled basis of every month for the first year and every 3 months for the second year. Primary end points were: (i) Combined end-stage renal disease (ESRD) and/or death and (ii) The presence of major or minor relapse during follow-up and secondary endpoint was the combination of ESRD and reduction of estimated glomerular filtration rate (eGFR) $\geq$ 50%.

Results: A total of 92 patients (M/F 39/53, mean age 59.1 $\pm$ 15 years) diagnosed with FNGN due to ANCA-associated vasculitis (AAV), 36 (39.1%) patients diagnosed with PR3-ANCA, 39 (42.4%) patients diagnosed with MPO-ANCA, and 17 (18.5%) patients diagnosed with ANCA(-) were included. Number of involved systems differed significantly between PR3-, MPO-ANCA, and ANCA(-), with only renal involvement in 3, 25.5, and 29% of patients, two systems involved in 33, 31, and 59% of patients, and $>$ 3 systems involved in 64, 43.5, and 12% of patients, respectively ($p = 0.002$). Histology classification revealed focal, crescentic, mixed, and sclerotic type in 14, 64, 19, and 3% of PR3-ANCA(+), 8, 28, 18, and 46% of MPO-ANCA, and 41, 29, 6, and 24% of ANCA(-), respectively ($p < 0.0001$). Primary end point of ESRD $\pm$ Death was reached in 11 (30.6%), 16 (41%), and 6 (35.5%) patients with PR3-ANCA(+), MPO-ANCA(+), and ANCA(-), respectively (p = NS); similarly, ESRD$\pm$ $>$ 50% eGFR reduction in 8 (22.2%), 15 (38.5%), and 5 (29.4%) patients, respectively (p = NS), meaning that patients with MPO-ANCA(+) showed a propensity to decline renal function. Rate of relapse was increased

in the presence of patients with PR3-ANCA(+), 14 (38.9%), 4 (11.8%), and 2 (10.3%) of patients with PR3-ANCA(+), MPO-ANCA(+), and ANCA(-), had at least one relapse during the two-year follow-up ($p = 0.006$).

Conclusion: Clinical phenotype and renal histology differ significantly between PR3-ANCA(+), MPO-ANCA(+), and ANCA(-) disease and FNGN; however, renal function outcome is similar, despite the increased rate of relapses in patients with PR3-ANCA(+).

Keywords: ANCA–associated vasculitis, crescentic, sclerotic, outcome, relapse, clinical phenotype

INTRODUCTION

Antineutrophil cytoplasmic antibodies (ANCAs)-associated vasculitis (AAV) is a group of autoimmune disorders affecting small and medium size vessels, characterized by necrotizing inflammatory reactions and their clinical consequences extent to a great spectrum of systems and organs including respiratory system, kidneys, skin, central and peripheral nervous system, gastrointestinal system, etc. Kidneys are affected at a high rate of 60–80% of cases, causing focal necrotizing glomerulonephritis (FNGN) and rapidly deteriorating kidney function in most cases, which may lead to end-stage renal disease (ESRD), if diagnosis and appropriate treatment are delayed (1).

The disease is characterized by the presence of antibodies directed mainly against proteinic targets located in the cytoplasmic granules of neutrophils. Recent studies have proved the pathogenic significance of ANCAs, during all the stages of disease progression, as they cause neutrophil stimulation, complement activation, aggregation of lymphocytes, macrophages, and platelets, which infiltrate vessel walls, causing necrotizing inflammation, destruction of small- and medium-sized vessels leading to necrosis of the tissues supplied (2, 3). Traditionally, AAVs include four clinical syndromes, namely, microscopic polyangiitis (MPA), granulomatosis with polyangiitis (GPA), eosinophilic GPA (EGPA), and renal limited disease (RLD) (4). Clinical syndromes have been described in an attempt to define distinct clinical entities in the spectrum of AAVs and distinguish patients in separate groups to better understand and approach pathogenesis and treatment. However, additional problems have raised; since, the description of clinical syndromes, substantial overlap between myeloperoxidase (MPO), GPA, and RLD do occur, in terms of type and clinical symptomatology of ANCA. Furthermore, the pathogenetic significance of ANCA, along with their importance in determining clinical presentation, disease activity, response to treatment, and predicting relapse, supports their use as biomarkers of the disease, but also forces investigators to rely on the presence and type of ANCA in order to discriminate different clinical phenotypes.

In this study, we evaluated the differences between MPO-ANCA(+), [proteinase 3 (PR3)-ANCA(+)], and ANCA(-) vasculitis in terms of clinical symptoms at presentation and severity of renal pathology; in addition, we prospectively assessed differences in response to treatment, the outcome of the disease, and rate of relapse in patients with necrotizing glomerulonephritis (NGN) due to AAV (5, 6).

PATIENTS AND METHODS

Patients

This prospective, longitudinal, and observational study included adult patients with NGN due to ANCA(+) vasculitis. Diagnosis in all the patients was based on renal biopsy findings, optical microscopy, and immunostaining (immunofluorescence and/or immunohistochemistry), showing pauci-immune NGN. Initial presentation of patients consisted of renal and/or extrarenal manifestations of ANCA(+) vasculitis; however, they were referred to our department, if they showed micro- or macroscopic hematuria, proteinuria, and/or renal function impairment.

Inclusion criteria were: age > 18 years old, renal biopsy at the time of presentation or not later than 1 month after presentation, induction, and maintenance treatment with the standard protocol, based on the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines. Only patients with newly diagnosed AAV were included and those patients with the known disease who presented with a relapse episode were excluded. Patients with evidence of systemic lupus erythematosus, rheumatoid arthritis, immunoglobulin A (IgA) vasculitis, cryoglobulinemia, abnormal immunoglobulin levels, antinuclear antibodies (ANAs), positive anti-glomerular basement membrane (GBM) antibodies or double-positive ANCA, and anti-GBM were excluded from this study. In addition, patients who had a recent or chronic infection and/or recent (<12 months) treatment with steroids or immunosuppressants were also excluded. All the patients signed the informed consent before participating. The study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of the Hippokration General Hospital, Thessaloniki, Greece, Approval Number 12/16.

Histology Assessment

All the renal biopsies were undergone assessment on both optical microscopies, using the routine stains including H&E, periodic acid–Schiff (PAS), periodic acid silver methenamine (PASM), and Masson's trichrome and immunostaining (immunofluorescence and/or immunohistochemistry) including immunoglobulins (IgA, IgG, and IgM), complement components (C3, C1q, and C4), fibrin, kappa, and lambda light chains.

The percentage of crescents, including cellular, fibrocellular, and fibrous crescents, was estimated on optical microscopy as well as the degree of tubule-interstitial infiltration, interstitial

TABLE 1 | Differences in the clinical symptoms and extrarenal manifestations between patients with myeloperoxidase-antineutrophil cytoplasmic antibody (MPO-ANCA)(+), proteinase 3 (PR3)-ANCA(+), and ANCA(-).

	MPO-ANCA (+)	PR3-ANCA (+)	ANCA (-)	*p*
N (%)	39 (42.4)	36 (39.2)	17 (18.4)	
Females	24 (61.5)	21 (58.3)	8 (47.1)	NS
Extrarenal manifestations				
Fever	17 (43.6)	12 (33.3)	2 (11.8)	0.05
Skin rash	8 (20.5)	8 (22.2)	3 (17.6)	NS
ENT	4 (10.3)	12 (33.3)	2 (11.8)	0.03
Lower respiratory system	16 (41)	26 (72.2)	6 (35.3)	0.007
CNS involvement	0	2 (5.6)	0	NS
Arthritis	9 (23.1)	18 (50)	4 (23.5)	0.03
Gastroinenstinal involvement	0	1 (2.8)	0	NS
HD dependent	20 (51.3)	8 (22.2)	4 (23.5)	0.01
BVAS score	15 (10–24)	17 (10–25)	14 (10–25)	NS
FFS score	2 (0–3)	2 (0-3)	2 (0–3)	NS

TABLE 2 | Laboratory findings in patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-) at the time of presentation.

	MPO-ANCA(+)	PR3-ANCA(+)	ANCA (-)	*p*
Age (yrs)	60.6 ±14.9	59.4 ±12.4	55.2 ±19	NS
WCC (K/μl)	9701 ±4260	10785 ±4024	10207 ±2456	NS
Neutrophils (%)	81 ±8.8	77 ±11.8	77 ±12.9	NS
Neutrophils	8042 ±3954	8604 ±4097	7965 ±2581	NS
Lymphocytes (%)	11.5 ±6.9	15.1 ±8.7	15.8 ±10.5	NS
Lymphocytes (K/μl)	952 ±612	1356 ±683	1503 ±842	0.02
NLR	11.5 ±8.5	7.7 ±4.5	9.1 ±8.3	NS
PLT (K/μl)*10^3	255 ±96	359 ±100	309 ±134	0.01
PLR	344 ±191	309 ±141	391 ±543	NS
Hb (g/dl)	10 ±1.4	11.1 ±1.5	10.09 ±1.6	NS
Ht (%)	30.6 ±4.5	33.5 ±4.5	30.5 ±5.1	NS
e-GFR (mL/min/1.73 m^2)	20.5 ±12.9	22.4 ±15.8	21.4 ±15.2	NS
Uprot (g/24 h)	1.6 ±1.1	1.7 ±1.6	3.5 ±3.3	NS

fibrosis, and tubular atrophy. The degree of tubule-interstitial infiltration was semiquantively assessed, based on a scale of 0–2 (where 0 was substituted for absence or mild, 1 for moderate, and 2 for severe). Assessment of the severity of tubular atrophy and interstitial fibrosis was based on the percentage of tubules with characteristic findings of atrophy and estimated on a scale of 0–3 (where 0 was substituted for no abnormalities, 1 for < 30%, 2 for 31–60%, and 3 for > 60% of tubules with atrophy).

Based on the criteria established by the Immunonephrology Working Group (IWGRP), all the biopsies were categorized into four classes: focal (≥50% normal glomeruli); crescentic (≥50% glomeruli with cellular crescents); mixed (<50% normal, <50% crescentic, and <50% globally sclerotic glomeruli); and sclerotic (≥50% globally sclerotic glomeruli).

Treatment Protocol

Treatment was based on the KDIGO guidelines; the same therapeutic protocol was applied to all the patients, including induction treatment with intravenous pulses of 500 mg prednisolone, given daily for 6 consecutive days, followed by oral prednisolone 1 mg/kg/day and intravenous (IV) cyclophosphamide at doses adjusted to age and renal function of the patient. Prednisolone doses were gradually reduced by 5 mg, every 2 weeks. Cyclophosphamide was administered at 10 pulses, followed by azathioprine or mycophenolate mofetil.

Plasmapheresis was performed, according to the KDIGO guidelines, in case of severe renal failure or pulmonary hemorrhage. Seven to ten plasma exchanges of 3,000 ml were performed in a day-by-day basis.

A kidney biopsy was performed either just before initiation of therapy or within 1 month, in cases with increased risk of bleeding or those requiring acute treatment.

Patients Follow-Up

Day of study entry was the day of starting treatment. Clinical parameters including extrarenal manifestations, namely, pulmonary, myoskeletal, central, and peripheral nervous system involvement, the Birmingham Vasculitis Activity Score

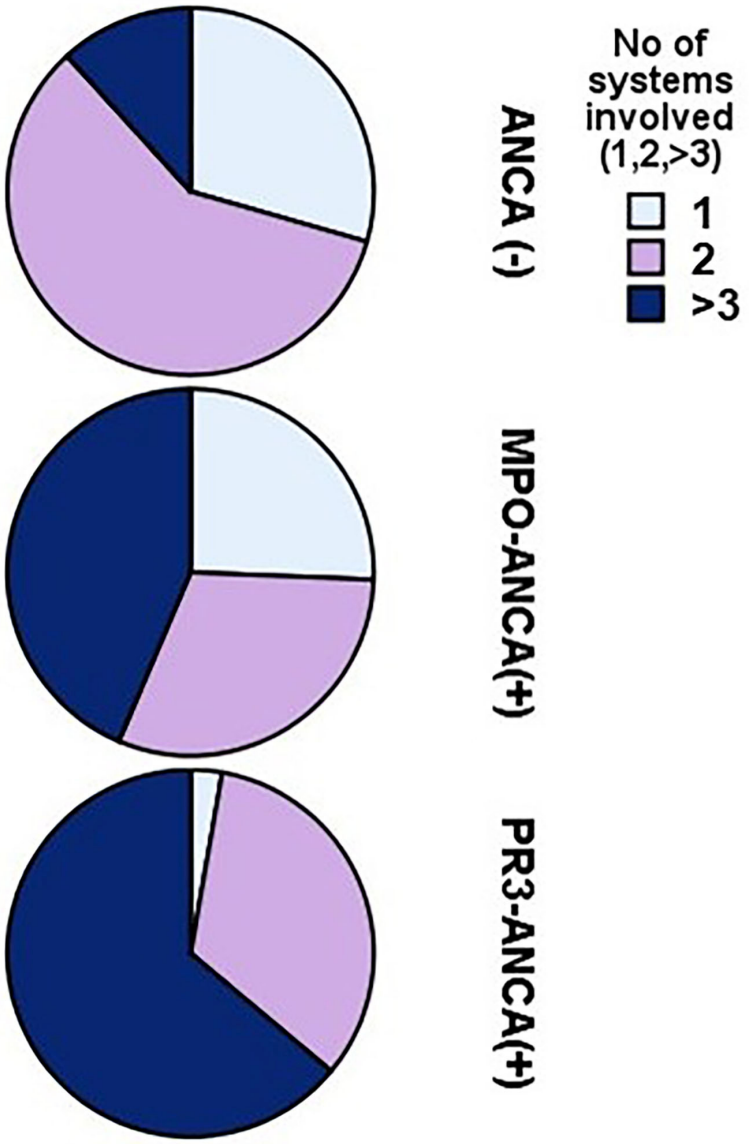

FIGURE 1 | Differences in the frequency of organ systems involvement according to the type of antineutrophil cytoplasmic antibody (ANCA).

(BVAS) and the Five-Factor Score-2009 (FFS-2009), laboratory investigation, including renal function, degree of proteinuria and serum levels of ANCA, and histological findings were estimated.

Patients were regularly followed-up on an outpatients basis, every month for the first year and every 3 months for the second year. In a case of a sudden event, such as relapse, infection, deterioration of renal function, or an acute event, patients were followed according to decision of the physician.

Parameters recorded at time of diagnosis were estimated glomerular filtration rate (eGFR), the BVAS and the FFS-2009 score, white cell count (WCC), neutrophil, lymphocyte, and platelets count, neutrophil/lymphocyte ratio (NLR) and platelet/lymphocyte ratio (PLR), and the need of hemodialysis (HD). During follow-up, eGFR was assessed every month for the first year and every 3 months for the second year and recorded at 3 (eGFRm3), 6 (eGFRm6), 12 (eGFRm12), and at the end of 24 months (eGFRm24). The slope of eGFR was estimated based on serial measurements of eGFR.

Primary endpoints were: (i) Combined event of ESRD and/or death and (ii) The presence of major or minor relapse of AAV during follow-up. As a secondary endpoint, the combination of ESRD and reduction of eGFR by $\geq$ 50% was defined. Patients were followed for 24 months; for those patients who reached ESRD and started on HD or patients who died, follow-up period lasted until the day of starting on HD or the day of death.

TABLE 3 | Numbers and percentage of patients presenting with 1, 2, or > 3 systems involved.

No of Exrarenal Manifestations*	ANCA (-)	PR3- ANCA	MPO-ANCA
1	5 (29)	1 (3)	10 (25.5)
2	10 (59)	12 (33)	12 (31)
>3	2 (12)	23 (64)	17 (43.5)

*Pearson chi-square test 17, $p = 0.002$, likelihood ratio 20.2, $p < 0.0001$.

Definitions

Estimated glomerular filtration rate was estimated by the formula Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI).

The BVAS version 3 score was calculated for all the patients. A total of 56 items, representing involvement of different systems or organs, including upper and lower respiratory, cardiovascular, abdominal, renal, and nervous system manifestations, cutaneous, mucous, and general indices, were organized into nine different groups.

The FFS-2009 score involved five factors and five points, such as age, renal insufficiency, cardiac involvement, gastrointestinal, and upper respiratory [ear-nose-throat (ENT)] manifestations. ENT signs were assessed in all the patients, regardless the presence or type of ANCA. The absence of ENT manifestations in presence of the rest parameters was considered as +1 point.

Major relapse was defined as life- or organ-threatening relapse after achieving complete or partial remission. Minor relapse was defined as an increase in disease activity after complete or partial remission, but not causing life- or organ-threatening complications.

Statistics

The Statistical Package for the Social Sciences (SPSS) (SPSS Incorporation, Chicago, Illinois, USA) version 25.0 for windows was used for the statistical analysis. Normality of the distribution for continuous variables was estimated by the Kolmogorov–Smirnov and the Shapiro–Wilk tests. Values of $p < 0.05$ (two-tailed) were considered statistically significant for all the comparisons.

Mean ± SD or medians and interquartile range (IQR) were used to describe data in normally distributed and nonparametric variables, respectively. In the same way, the Student's *t*-test for nonpaired or the ANOVA test and the Mann–Whitney *U* test or the Wilcoxon signed-rank test, respectively, were performed to compare differences between groups.

RESULTS

Characteristics of Patients at Time of Diagnosis

We included 92 patients, M/F 39/53, mean age 59.1 ± 15 years, who were diagnosed with FNGN due to AAV in the period 1/2016–1/2019. A total of 39 patients (42.4%) had MPO-ANCA, 36 (39.1%) patients had PR3-ANCA, and 17 (18.5%) patients were ANCA negative.

At the time of diagnosis, median levels of proteinuria Urine protein (Upr) were 1.49 (0.2–11) g/24 h and eGFR was 13.8 (6.15–98.2) mg/dl/1.73 m^2. A total of 32 patients (34.8%) were dialysis-dependent at presentation and eGFR in the rest 60 patients was 22 (10.8–98.2) mg/dl/1.73 m^2.

Differences According to the Type of ANCA

Clinical characteristics, manifestations of vasculitis, disease activity and laboratory findings, and differences between ANCA types are shown in **Tables 1**, **2**.

Extrarenal Manifestations

The number of extrarenal manifestations was estimated for patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-) and results are given in **Figure 1** and are shown in detail in **Table 3**.

The presence of PR3-ANCA was followed by advanced extrarenal organ involvement. In patients with PR3-ANCA(+), only 3% presented with clinical symptoms restricted to kidneys, while 64% had more than three systems involved, compared to patients with 43.5% in MPO-ANCA(+) and only 12% in ANCA(-).

Renal Biopsy Findings

From the whole cohort of patients, 74 (80.4%) patients had focal necrosis on renal biopsy and 14 (15.2%) patients had endocapillary hyperplasia. A median number of glomeruli with crescent formation was estimated to be 56.25 (0–100), 23 (0–100) of them were cellular, 12.38 (0–100) were fibrocellular, and 8.37 (0–50) were fibrous crescents. Severity of interstitial fibrosis was estimated as 0 in 11 (12%), 1 in 50 (54.3%), and 2 in 31 (33.7%) patients; inflammatory infiltration of interstitium was graded as 0 in 6 (6.5%), 1 in 45 (49%), and 2 in 41(44.5%) patients.

When histological findings were classified into four different classes (focal, crescentic, mixed, and sclerotic) according to the IWGRP (7), we noticed a significant difference between patients with ANCA(-), MPO-ANCA(+), and PR3-ANCA(+), as shown in **Figure 2**. The crescentic type had a significantly increased incidence in patients with PR3-ANCA(+), almost 64% of them presented with the crescentic type compared to <30% in both the patients with MPO-ANCA(+) and ANCA(-). Instead, the sclerotic type had a privilege in patients with MPO-ANCA(+), 46% of whom had a sclerotic type, compared to patients with 24% of ANCA(-) and only 3% of PR3-ANCA(+).

Renal Function Outcome

Changes in eGFR for the whole cohort of patients and separately for MPO-ANCA(+), PR3-ANCA(+), and ANCA(-) are shown in **Table 4** and **Figure 3**.

Although there were differences in renal function at the time of diagnosis and during follow-up between patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-), these differences could not reach statistical significance.

The primary endpoint of ESRD ± Death was reached in 16 (41%), 11 (30.6%), and 6 (35.5%) patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-), respectively ($p =$

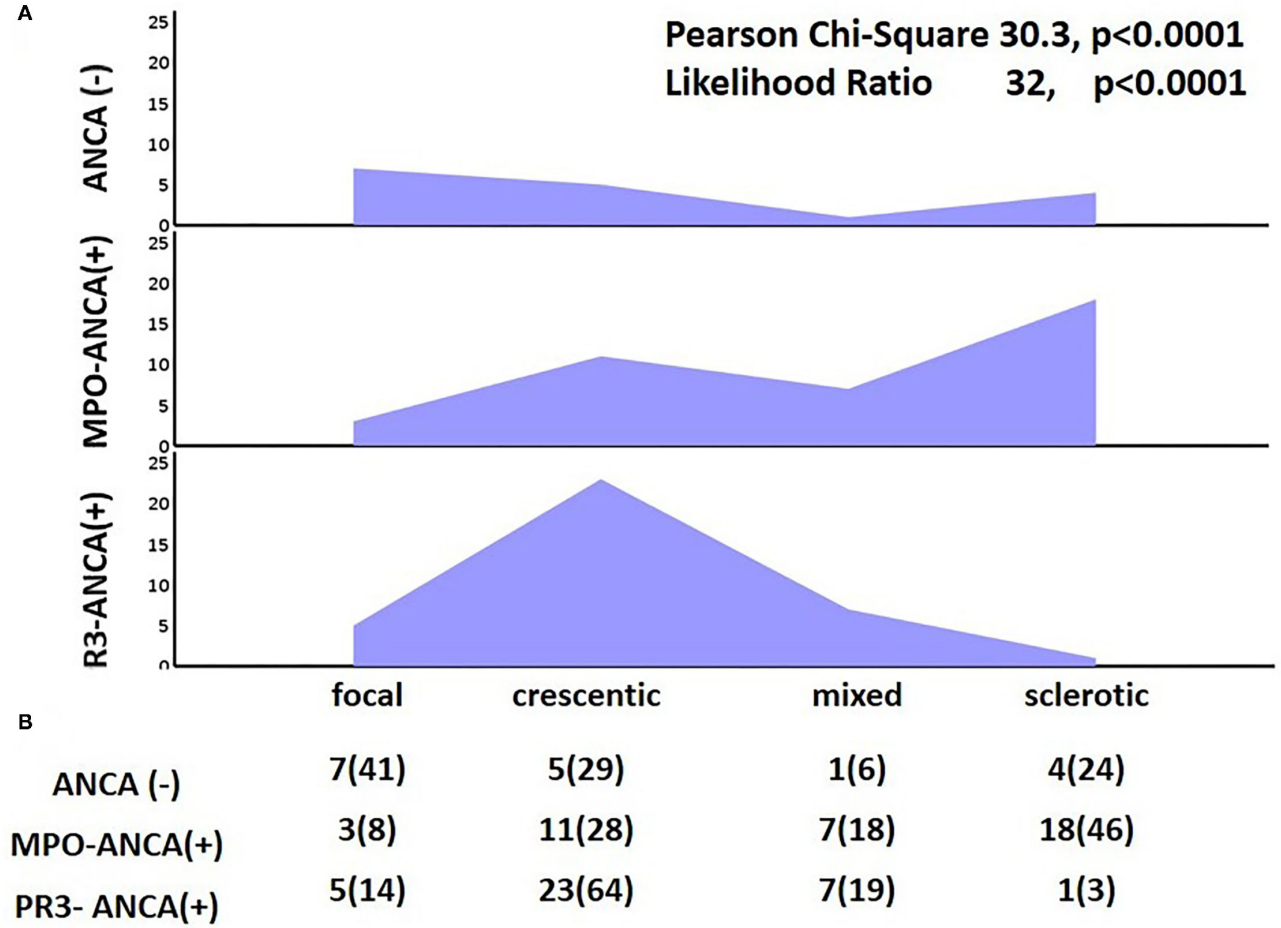

FIGURE 2 | Histological classification of renal biopsy findings; **(A)** depicts the ratio of focal, crescentic, mixed, and sclerotic changes in patients with ANCA(-), myeloperoxidase (MPO)-ANCA(+), and proteinase 3 (PR3)-ANCA(+) and **(B)** describes number and percentage of patients with the above changes.

TABLE 4 | Renal function outcome in the three groups of patients such as MPO-ANCA(+), PR3-ANCA (+), and ANCA(-).

	e-GFR (m0)	e-GFR (m3)	e-GFR (m6)	e-GFR (m12)	e-GFR (m24)	*p*
MPO(+) ANCA	20.5 ± 22.9	28.5 ± 24.2	32.1 ± 26.8	32.9 ± 29.4	31.7 ± 28.7	0.001
PR3(+) ANCA	22.4 ± 15.8	34.7 ± 22	38.2 ± 24.9	38.7 ± 25.8	36.5 ± 26.2	0.003
ANCA (-)	21.4 ± 15.2	28.2 ± 17.8	31.3 ± 21.6	32.2 ± 22.5	34.5 ± 25.8	0.05

NS); similarly, the secondary endpoint of ESRD± > 50% reduction in eGFR was reached by 15 (38.5%), 8 (22.2%), and 5 (29.4%) patients, respectively (p = NS), meaning that there was a tendency of patients with MPO-ANCA(+) to decline renal function, though not reaching statistical significance. However, with respect to the relapse rate, there was a clear preponderance of patients with PR3-ANCA(+). The percentage of patients who had at least one, major or minor, relapse during the 2-year follow-up was 14 (38.9%), 4 (11.8%), and 2 (10.3%) in patients with PR3-ANCA(+), MPO-ANCA(+), and ANCA(-) ($p = 0.006$).

Impact of Histology in the Outcome of Renal Function

Outcome of renal function was significantly associated with histological classification. Thus, primary end point was reached in 3/15 (20%), 10/39 (25.6%), 3/15 (20%), and 17/23 (73.9%) patients with focal, crescentic, mixed, and sclerotic type on renal biopsy, respectively ($p < 0.0001$). Similarly, secondary end point was reached in 2/15 (13.3%), 8/39 (20.5%), 2/15 (13.3%), and 17/23 (73.9%) patients, respectively ($p < 0.0001$).

However, there were no significant differences when we compared outcomes of patients in the same histological type

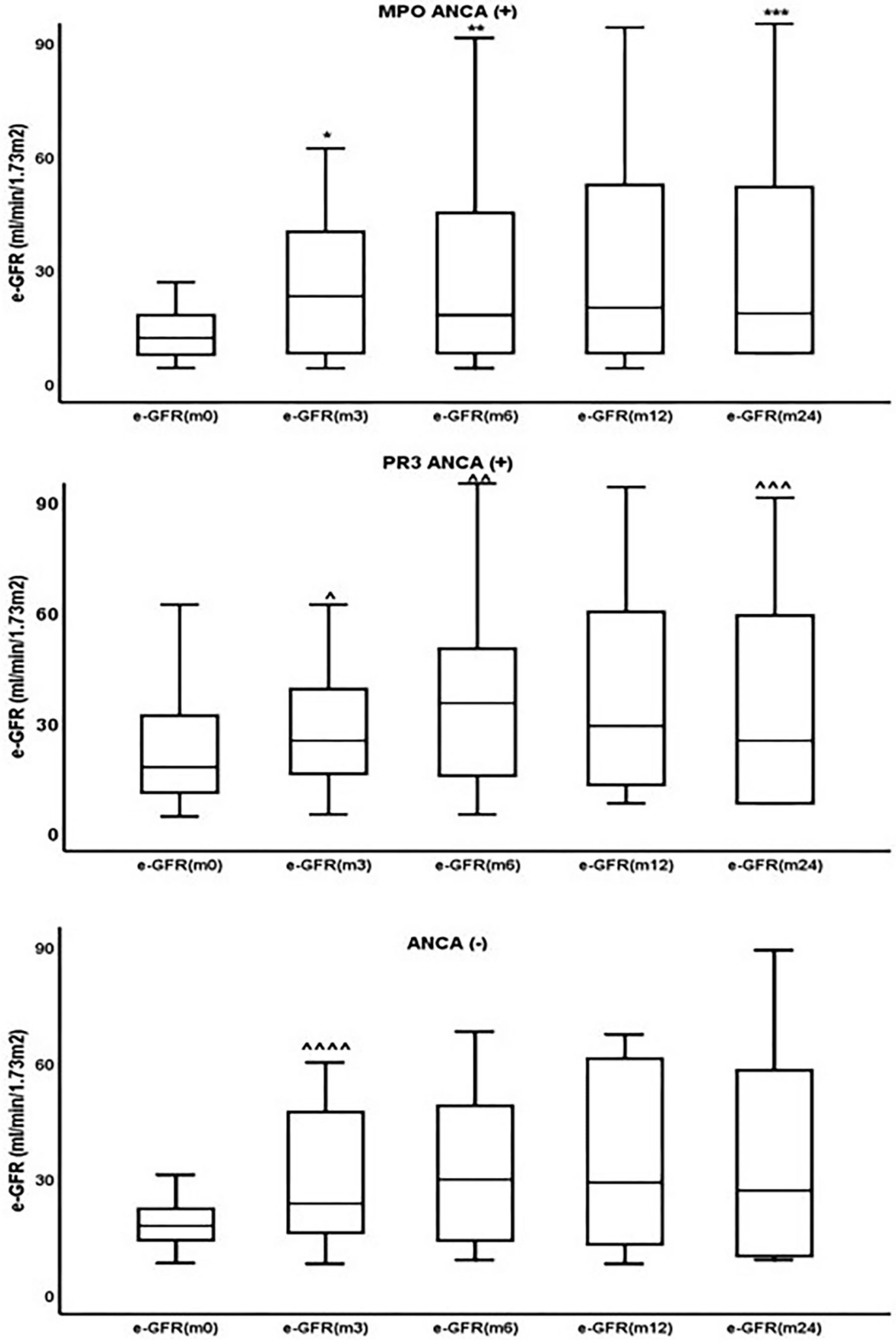

FIGURE 3 | Changes in estimated glomerular filtration rate (eGFR) during follow-up in the three groups of patients. *p [eGFR (m3) vs. eGFR (m0)] = 0.004, **p [eGFR (m6) vs. eGFR (m3)] = 0.01, ***p [eGFR (m24) vs. eGFR (m12)] = 0.004, ^p [eGFR (m3) vs. eGFR (m0)] = 0.001, ^^p [eGFR (m6) vs. eGFR (m3)] = 0.04, ^^^p [eGFR (m24) vs. eGFR (m12)] < 0.0001, ^^^^p [eGFR (m3) vs. eGFR (m0)] = 0.05.

according to ANCA type, probably because of the relatively small number of patients.

DISCUSSION

During the last few years, there is accumulated evidence, which supports the use of ANCA specificity rather than the use of clinical syndromes, MPA, GPA, EGPA, and RLD, to categorize the AAVs (6, 8). We performed a prospective study in patients with renal involvement, in particular FNGN, due to AAV, to investigate whether specific clinical presentation, histology, and outcome of MPO-ANCA(+), PR3-ANCA(+), and ANCA(-) diseases support their definition as different entities.

In this study, 92 patients with biopsy-proven NGN due to AAV were included. Thirty nine of them, 42% were MPO-ANCA(+), 36 (39%) were PR3-ANCA(+), and 17 (19%) were ANCA(-). Epidemiologically, AAV is a rare autoimmune disease with a high mortality risk, if diagnosis is delayed. The incidence of the disease is estimated to be 13–30 cases per million population in North America and Europe (9, 10). Geographically, there seems to be a difference in the incidence of the two types of AVV, with PR3-ANCA(+) disease being more prevalent and northern parts of the world and MPO-ANCA(+) predominating in the south, southern Europe, and Asia (11). In agreement with these finding, this study, which included Caucasian population, mainly from Southern Europe, proved a slightly higher prevalence of MPO-ANCA, compared to PR3-ANCA and ANCA(-).

Patients with PR3-ANCA showed an increased frequency of extrarenal manifestations at presentation including involvement of upper and lower respiratory system and arthritis. On the other hand, MPO-ANCA-associated vasculitis often presented with advanced renal impairment, with more than 50% of patients with MPO-ANCA(+) requiring hemodialysis at time of diagnosis, compared to only 22 and 23.5% of patients with PR3-ANCA(+) and ANCA(-), respectively.

Our findings support recent evidence that the two types of ANCA-associated vasculitis may represent two distinct diseases, accompanied by rather fewer common and more divergent characteristics. Indeed, in this study, MPO-ANCA(+) vasculitis, apart from the impaired renal function, seemed to be placed more closely to ANCA(-) vasculitis rather than PR3-ANCA(+), in terms of the rest clinical and laboratory characteristics.

The two diseases have differences not only in the frequency of specific organ involvement, but also in the way precise organs are affected. For instance, the upper respiratory system is more frequently affected in PR3-ANCA(+) and kidneys predominated in MPO-ANCA(+) vasculitis (12, 13). Moreover, patients with MPO-ANCAs experience more severe renal disease at presentation, which is due to the increased frequency of chronic lesions in renal biopsy (13). Correspondingly, they express more severe pulmonary interstitial fibrosis, whereas lung involvement in PR3-ANCA(+) disease usually presents with cavitary lesions or nodules. Alveolar hemorrhages equally found in both the ANCA types, although other manifestations, such as destructive nose, ear, and throat, seem to be more common in patients with PR3-ANCA(+) (5, 14). In our patients, we described significant differences in the renal histology, crescentic type was prominent in PR3-ANCA(+), while the sclerotic type of AVV was the predominant type among patients with MPO-ANCA(+).

Different clinical phenotypes and different histology phenotypes may represent diversity in disease pathogenesis, depending on the presence and type of ANCA. *In-vivo* studies have proved the pathogenic significance of both the MPO- and PR3-ANCA through the initiation and maintenance of inflammatory reactions (15–18). Even more importantly, the genetic background of AVV has also been proved, with studies confirming the association of AVVs with the major histocompatibility complex (MHC) and specifically with gene variants in HLA-DPA1 and DPB1 for PR3-ANCA-AAV and HLA-DQA2 and DQB1 for MPO-ANCA-AAV (19, 20). The above studies confirmed that MPA and GPA are genetically distinct autoimmune diseases, although the genetic profile seems to be much stronger associated with ANCA specificity rather than the clinical phenotype. Beyond the genetic basis, further issues are still arising, in the use of clinical AAV syndromes, namely, MPA, GPA, and EGPA. There is a clear overlap between them, as they share common clinical features and their classification is rather subjective and based to initial disease features (5, 19, 20).

There is a decent percentage of patients, around 30%, that present with clinicopathological features of AAV, but their ANCA titles are negative. A variety of reasons may be implicated including differences in ANCA detection methods, the timing of testing, or the presence of antigens not yet determined (21). Patients with ANCA(-) often have a renal limited disease rather than generalized multisystemic disease, lesser extrarenal manifestations, and are of younger age compared to ANCA(+) (22). However, to the best of our knowledge, for this group of patients is limited, since it is a group that is often excluded from the studies and, thus, more studies need to be conducted to reach assertive results and information.

Although there were differences in renal function at the time of diagnosis and during follow-up between patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-) and there also was a significant difference in the need for dialysis in the group of patients with MPO-ANCA and these differences could not reach statistical significance. Our primary endpoint of either ESRD and/or death was reached in 16 (41%), 11 (30.6%), and 6 (35.5%) patients with MPO-ANCA(+), PR3-ANCA(+), and ANCA(-), respectively, and the secondary endpoint of ESRD$\pm$ $>$ 50% reduction in eGFR in 15 (38.5%), 8 (22.2%), and 5 (29.4%) patients, respectively, although, did not manage to reach statistical significance, but showed a propensity of patients with MPO-ANCA(+) to impaired renal function. There are several cohorts that showed a higher mortality rate in patients with MPO-ANCA than those with patients with PR3-ANCA, but this difference is usually not statistically significant after adjustment for age, mostly because there seems to be a 10-year discrepancy between the two groups of patients; PR3-ANCA(+) vasculitis is common among 45–55-year-old patients, while MPO-ANCA(+) vasculitis is common in 60–65-year-old patients (13, 23, 24). Overall, both the renal and mortality prognosis seems to be

the same for each group of patients and is independent of the ANCA type (5).

With respect to the relapse rate, however, we found that there was a clear dominance in patients with PR3-ANCA(+), with the frequency of patients who had at least one relapse during the 2 years follow-up, reaching to more than four times compared to patients with MPO-ANCA(+) and ANCA(-). Our results were in accord to the literature, as the most important risk factor for relapse of the disease seems to be the presence of PR3-ANCA(+), which is accompanied by a 2-fold higher risk for relapse (5, 25). Interestingly, MPO-ANCA(+) disease has the propensity to relapse later and this could possibly explain the increased differences in relapse rate in this study. The high relapse rate of patients with PR3-ANCA vs. MPO-ANCA does not seem to have an impact on their long-term survival (26). This could be explained by the fact that patients with MPO-ANCA(+) have poorer renal outcomes, worse renal disease, they are more HD dependent, and have more chronic lesions in kidney biopsy than MPO-ANCA(+) vasculitis (27).

In this study, we confirm the distinct clinical phenotypes of MPO-ANCA(+) and PR3-ANCA(+) disease; we describe differences in histology, but, in addition, we point out the similarities between MPO-ANCA(+) and ANCA(-) vasculitis. Response to treatment and outcome of renal function were similar in the three groups, regardless the type of ANCA. PR3-ANCA(+), however, seemed to be more "aggressive," presenting with a great spectrum of organ involvement and relapsing early and frequently during follow-up. MPO-ANCA(+) disease, on the other hand, is still and quiet and when it is diagnosed, it has usually caused irreversible damage with advanced fibrosing lesions in renal biopsy. ANCA(-) disease acts as a discrete disease, which definitely reminds MPO-ANCA(+) disease. However, although our findings come from a prospective close follow-up of patients, certainly more longitudinal studies are needed to establish the distinction of clinical entities based on the presence of ANCA types, rather than the clinical phenotypes.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Ethics Committee of the Hippokration General Hospital, Thessaloniki, Greece, Approval Number 12/16. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

KB, MS, and AP contributed to conception and design of the study. KB wrote the first draft of the manuscript. MS supervised the work and revised the paper. KB and SK organized the database. CN assessed renal biopsies. GL performed the statistical analysis. ZM and FI wrote sections of the manuscript. AF assessed patients immunological results. All authors contributed to manuscript revision, read, and approved the submitted version.

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Development of a Risk Model for Predicting Microalbuminuria in the Chinese Population Using Machine Learning Algorithms

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Objective: Microalbuminuria (MAU) occurs due to universal endothelial damage, which is strongly associated with kidney disease, stroke, myocardial infarction, and coronary artery disease. Screening patients at high risk for MAU may aid in the early identification of individuals with an increased risk of cardiovascular events and mortality. Hence, the present study aimed to establish a risk model for MAU by applying machine learning algorithms.

Methods: This cross-sectional study included 3,294 participants ranging in age from 16 to 93 years. R software was used to analyze missing values and to perform multiple imputation. The observed population was divided into a training set and a validation set according to a ratio of 7:3. The first risk model was constructed using the prepared data, following which variables with $P < 0.1$ were extracted to build the second risk model. The second-stage model was then analyzed using a chi-square test, in which a $P \geq 0.05$ was considered to indicate no difference in the fit of the models. Variables with $P < 0.05$ in the second-stage model were considered important features related to the prevalence of MAU. A confusion matrix and calibration curve were used to evaluate the validity and reliability of the model. A series of risk prediction scores were established based on machine learning algorithms.

Results: Systolic blood pressure (SBP), diastolic blood pressure (DBP), fasting blood glucose (FBG), triglyceride (TG) levels, sex, age, and smoking were identified as predictors of MAU prevalence. Verification using a chi-square test, confusion matrix, and calibration curve indicated that the risk of MAU could be predicted based on the risk score.

Conclusion: Based on the ability of our machine learning algorithm to establish an effective risk score, we propose that comprehensive assessments of SBP, DBP, FBG, TG, gender, age, and smoking should be included in the screening process for MAU.

Keywords: microalbuminuria, risk score, risk factors, machine learning, predicting

INTRODUCTION

Microalbuminuria (MAU) is defined as a urinary albumin excretion of 20–200 mg/L in a spot urine test or 30–300 mg in a 24-h urine collection test (1). The presence of MAU represents an early manifestation of general endothelial damage, which can occur secondary to diabetes, hypertension, and coronary heart diseases (2, 3). Research has demonstrated that MAU is closely associated with stroke, myocardial infarction, coronary artery disease, and all-cause mortality (4). Several studies have also indicated that MAU is predictive of vascular disease, diastolic dysfunction, congestive heart failure, and hypertension (5–7). Hence, clinical screening and early identification of MAU remains especially important.

Advancements in proteomics technology such as protein separation, biological mass spectrometry, and bioinformatics have decreased the difficulty of examining proteome expression (8). Despite these advancements, there are still many drawbacks in the detection of urine albumin (8). The gold standard in chronic kidney disease (CKD) screening is the 24-h urine collection test; however, this method is difficult to implement on a large scale due to its inconvenience (2).

Therefore, in the present study, we aimed to establish and validate a risk model for early prediction of MAU using machine learning algorithms rather than the results of 24-h urine microalbumin tests. Application of risk scores derived using such a model would be more convenient for the monitoring and follow up of patients at higher risk for MAU.

METHODS

Study Population

This cross-sectional study was performed between June 2011 and January 2012 and included participants randomly selected using a clustered sampling technique (9), with probabilities proportionate to the size of the population in each cluster. All participants were from Ningde City in Fujian province in southeast China. Overall, 3,294 Chinese (age: 16–93 years) participants who had no cognitive dysfunction and were not pregnant participated in the survey. MAU was defined as a urinary albumin excretion of 20–200 mg/L and was assessed using a spot urine test (1, 4). The exclusion criteria were as follows: history of type-1 diabetes mellitus (DM), history of kidney disease or urinary albumin excretion ≥200 mg/L, and pregnancy. The study was performed in accordance with the Declaration of Helsinki and approved by the Ethics Committee of Fujian Provincial Hospital (approval No. K2009-12-020), and written informed consent was obtained from each participant. All investigators who were unaware of the study's aims or the characteristics of the participants received special training before the investigation. **Figure 1** shows a flowchart describing patient selection.

Data Collection

All participants were required to complete a standard self-reported questionnaire comprising 10 questions addressing age, sex, personal and family medical history, smoking and drinking habits, and so on.

Weight, height, and waist circumference (WC) were measured to the nearest 0.1 kg and 0.1 cm, respectively, by experienced nurses, with patients wearing light clothing and no shoes. WC was measured at the middle point between the costal margin and iliac crest. Systolic and diastolic blood pressures (SBP and DBP) were both measured twice using a standard OMRON auto-electronic sphygmomanometer, and the mean of the two readings was used for analysis.

Blood samples were collected after an 8- to 12-h overnight fast and were stored at −20°C until analysis. Participants were provided with oral and written instructions on the collection of urine samples and advised to postpone urine collection in case of urinary tract infection, fever, or menstruation, and to avoid heavy exercise as much as possible during the collection period. The blood samples were evaluated at the Laboratory of Ningde Municipal Hospital. Each blood sample was independently assessed by two qualified examiners. Blood glucose levels were determined using the glucose oxidase method (Sclavo, Siena, Italy). The automatic colorimetric method (Hitachi, Boehringer Mannheim) was used to determine total cholesterol (TC), total triglyceride (TG), and high-density lipoprotein cholesterol (HDL-C) levels. Low-density lipoprotein cholesterol (LDL-C) levels were calculated using the Friedewald formula.

Type-2 DM was defined as a fasting blood glucose (FBG) ≥7.0 mmol/L or 2-h postprandial blood glucose (PBG) ≥11.1 mmol/L, previous diagnosis of type-2 DM, or use of hypoglycemic medications (10). Hypertension was defined as SBP ≥140 mmHg and/or DBP ≥90 mmHg, or use of antihypertensive medications (11). The Homeostatic Model Assessment (HOMA) values for β-cell function and insulin resistance (IR) were determined using the following simplified equations: HOMA-IR = [fasting plasma insulin (FPI) × FPG]/22.5; HOMA-β = (20 × FPI)/(FPG−3.5) (12, 13). The following were used as indices of insulin secretion in the current study: insulinogenic index = (Ins_{30}-Ins_0)/($Gluc_{30}$-$Gluc_0$), where Ins_y and Glu_y represent values at time (y:min) during the oral glucose tolerance test (OGTT) (14, 15).

Statistical Analysis

All calculations were performed using R software (version 3.6.3 GUI 1.70 EI Capitan build, 7735).

The "vim" package for R software was used to analyze missing values and visualize the data. The "mice" package was used to perform multiple imputation on missing values (m = 5, method = "pmm," maxit = 100, seed = 1,234). The imputed data and their distribution in the original dataset were analyzed and visualized using the "lattice" package.

The observed population was divided into a training set and a validation set according to a ratio of 7:3. The "glm" package

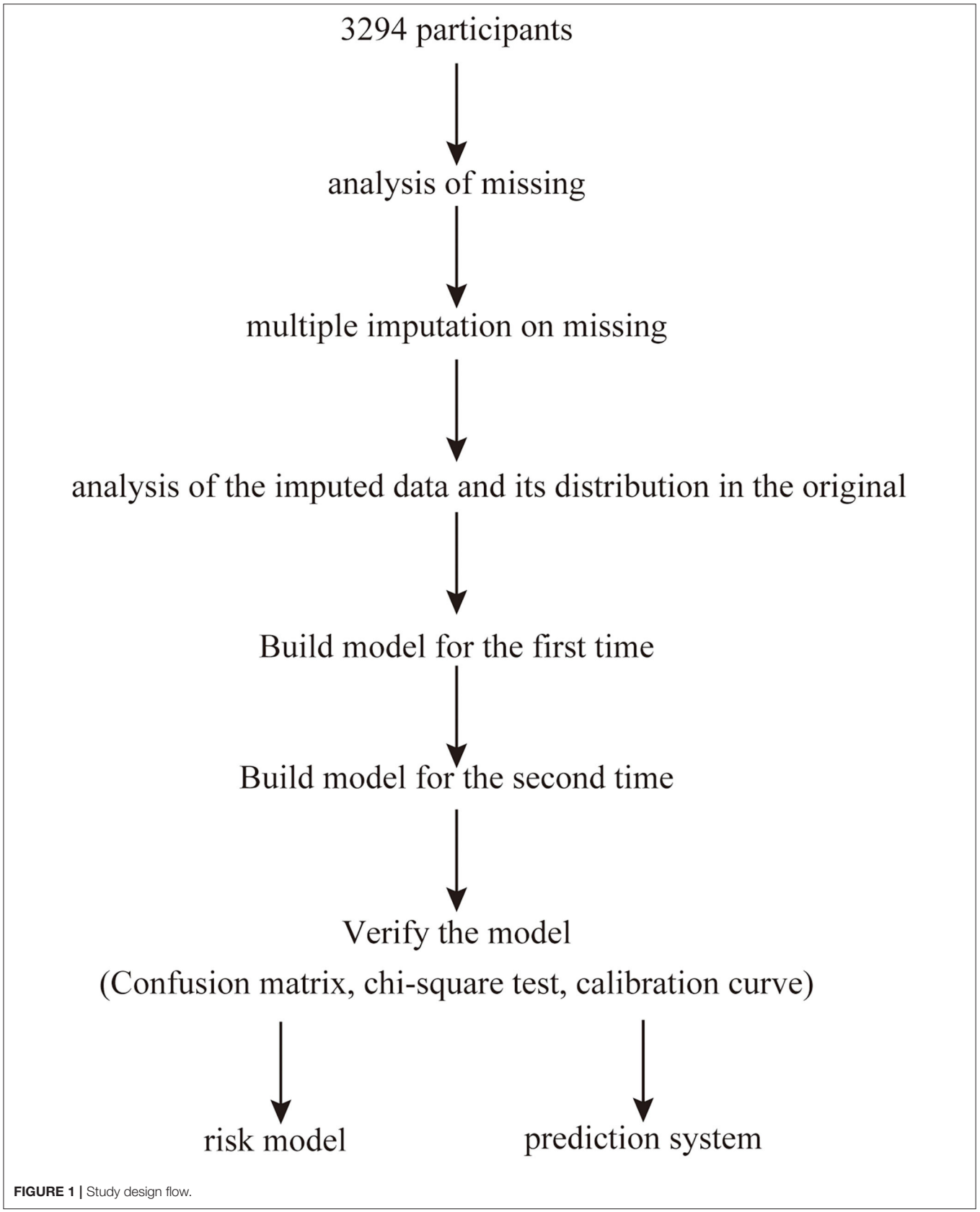

FIGURE 1 | Study design flow.

was used to build the first risk model using the prepared data. Then, variables with $P < 0.1$ were extracted to build the second risk model, also using the "glm" package. A chi-square test of the second-stage model was performed using the "anova" package, and a $P \geq 0.05$ was considered to indicate no difference in the fit of the model. A confusion matrix was used to verify the accuracy of the model, and a calibration curve was constructed using the "calibrate" package. Values of x closer to y in the calibration curve were considered to indicate better calibration of the model. Variables with $P < 0.05$ in the second-stage model were regarded as important features related to the prevalence of MAU. Graphical representations of the results were drawn using the "forestplot" package, and the risk score was established using a nomogram.

TABLE 1 | Characteristics of the study participants.

Parameters	Men (%)	Women (%)
Urinary albumin	444 (37.6%)	736 (62.4%)
Drinking	636 (73.4%)	231 (26.6%)
Smoker	$n = 668$ (97.1%)	$n = 20$ (2.9%)
Hypertension	$n = 480$ (40.3%)	$n = 711$ (59.7%)
Diabetes	$n = 174$ (44.5%)	$n = 217$ (55.5%)

RESULTS

Participant Characteristics

The enrolled participants were categorized based on urinary albumin levels, gender, presence of hypertension/diabetes, and smoking and drinking habits. The study population comprised 3,294 study participants [men: 1,294 (39.3%); women: 2,000 (60.7%)]. The characteristics of the participants are shown in **Tables 1**, **2**. A visual depiction of the distribution of these characteristics is shown in **Figure 2**.

Analysis of Missing Values

Twenty-eight observation indices were analyzed for missing values. The insulinogenic index represented the index with the most missing values, accounting for 10% ($n = 330$), followed by HOMA-IR and HMOA-β, which accounted for <10%. An analysis of trends in the distribution of missing values indicated that they were randomly distributed, conforming to the missing-at-random (MAR) assumption (**Figures 3A,B**). The "mice" package was used to perform multiple imputation on data with missing values (m = 5, method = "pmm," maxit = 100, seeds = 1,234). The imputed data and their distribution in the original dataset are shown in **Figure 3C**.

TABLE 2 | Laboratory data for the study participants.

	Total	NMAU	MAU
Ins_0 min (mmol/L)	7.12 (4.95 ~ 9.98)	7.14 (4.93 ~ 10.03)	7.09 (4.97 ~ 9.85)
Ins_30 min (mmol/L)	39.76 (24.07 ~ 63.43)	40.78 (25.25 ~ 63.82)	38.82 (22.20 ~ 62.35)
Ins_120 min (mmol/L)	29.88 (17.26 ~ 51.79)	28.90 (16.77 ~ 48.80)	31.80 (18.42 ~ 55.62)
Height (cm)	161 (155 ~ 167)	161 (156 ~ 167)	160 (155 ~ 167)
Weight (kg)	60 (53 ~ 67)	59.2 (53 ~ 67)	60.5 (54 ~ 69)
BMI	23.1 (21.0 ~ 25.4)	22.9 (20.7 ~ 25.1)	23.6 (21.4 ~ 26)
Waistline (cm)	79 (72 ~ 86)	78 (72 ~ 85)	80 (74 ~ 88)
Hipline (cm)	93 (89 ~ 98)	93 (89 ~ 97)	94 (89 ~ 98)
MSBP (mmHg)	120 (110 ~ 134)	118 (108 ~ 130)	123 (110 ~ 140)
MDBP (mmHg)	78 (70 ~ 85)	75 (70 ~ 82)	80 (70 ~ 86)
rHR (per min)	75 (69 ~ 83)	75 (68 ~ 82)	76 (69 ~ 83)
Bg_0 min (mmol/L)	4.87 (4.45 ~ 5.36)	4.82 (4.42 ~ 5.29)	4.93 (4.50 ~ 5.55)
Bg_30 min (mmol/L)	8.50 (7.24 ~ 10.00)	8.38 (7.10 ~ 9.80)	8.70 (7.43 ~ 10.38)
Bg_120 min (mmol/L)	5.88 (4.72 ~ 7.67)	5.70 (4.61 ~ 7.15)	6.20 (4.97 ~ 8.47)
Cholesterol (mmol/L)	4.79 (4.14 ~ 5.45)	4.73 (4.10 ~ 5.36)	4.86 (4.21 ~ 5.60)
Triglyceride (mmol/L)	1.17 (0.8 ~ 1.8)	1.11 (0.77 ~ 1.69)	1.28 (0.88 ~ 1.99)
HDL (mmol/L)	1.25 (1.02 ~ 1.50)	1.27 (1.04 ~ 1.50)	1.22 (1.00 ~ 1.48)
LDL (mmol/L)	2.83 (2.26 ~ 3.44)	2.79 (2.21 ~ 3.39)	2.90 (2.31 ~ 3.52)
Age (years)	46 (35 ~ 57)	44 (34 ~ 55)	48 (38 ~ 61)
HOMA-IR	1.56 (1.08 ~ 2.26)	1.54 (1.08 ~ 2.22)	1.60 (1.11 ~ 2.36)
HOMA-β	103.42 (63.76 ~ 168.21)	109.92 (68.19 ~ 175.45)	95.43 (56.77 ~ 153.86)
Insulinogenic-index	9.47 (4.71 ~ 17.36)	9.81 (5.03 ~ 17.69)	8.78 (4.26 ~ 16.64)

NMAU, No microalbuminuria; MAU, microalbuminuria; Ins, Insulin releasing test; BMI, Body Mass Index; MSBP, mean systolic blood pressure; MDBP, mean diastolic blood pressure; rHR, resting heart rate; Bg, blood glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, Homeostasis Model Assessment- insulin resistance; HOMA-β, Homeostasis Model Assessment of β-cell function.

Data are shown as the median (interquartile range).

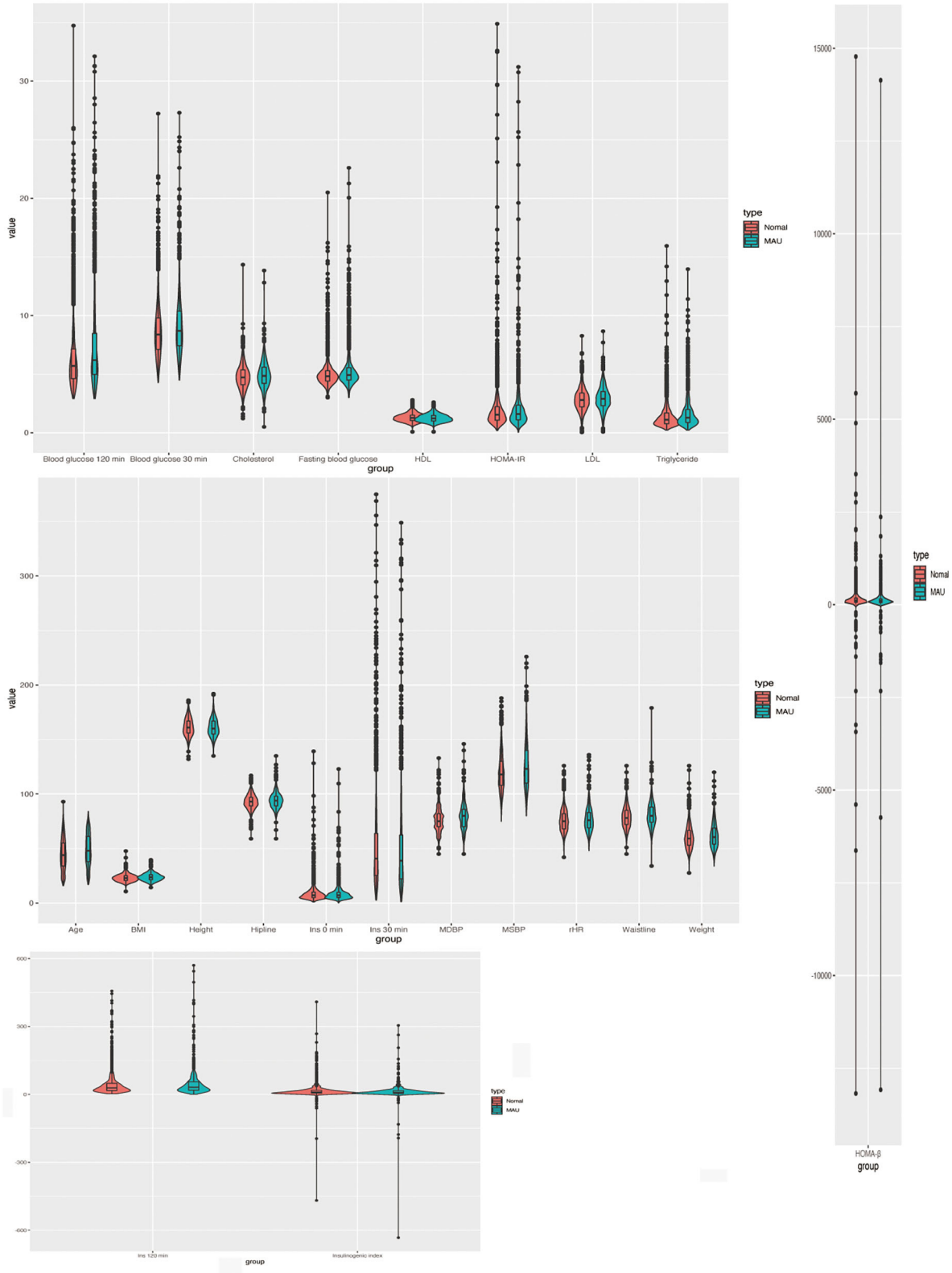

FIGURE 2 | The distribution of characteristic of the participants.

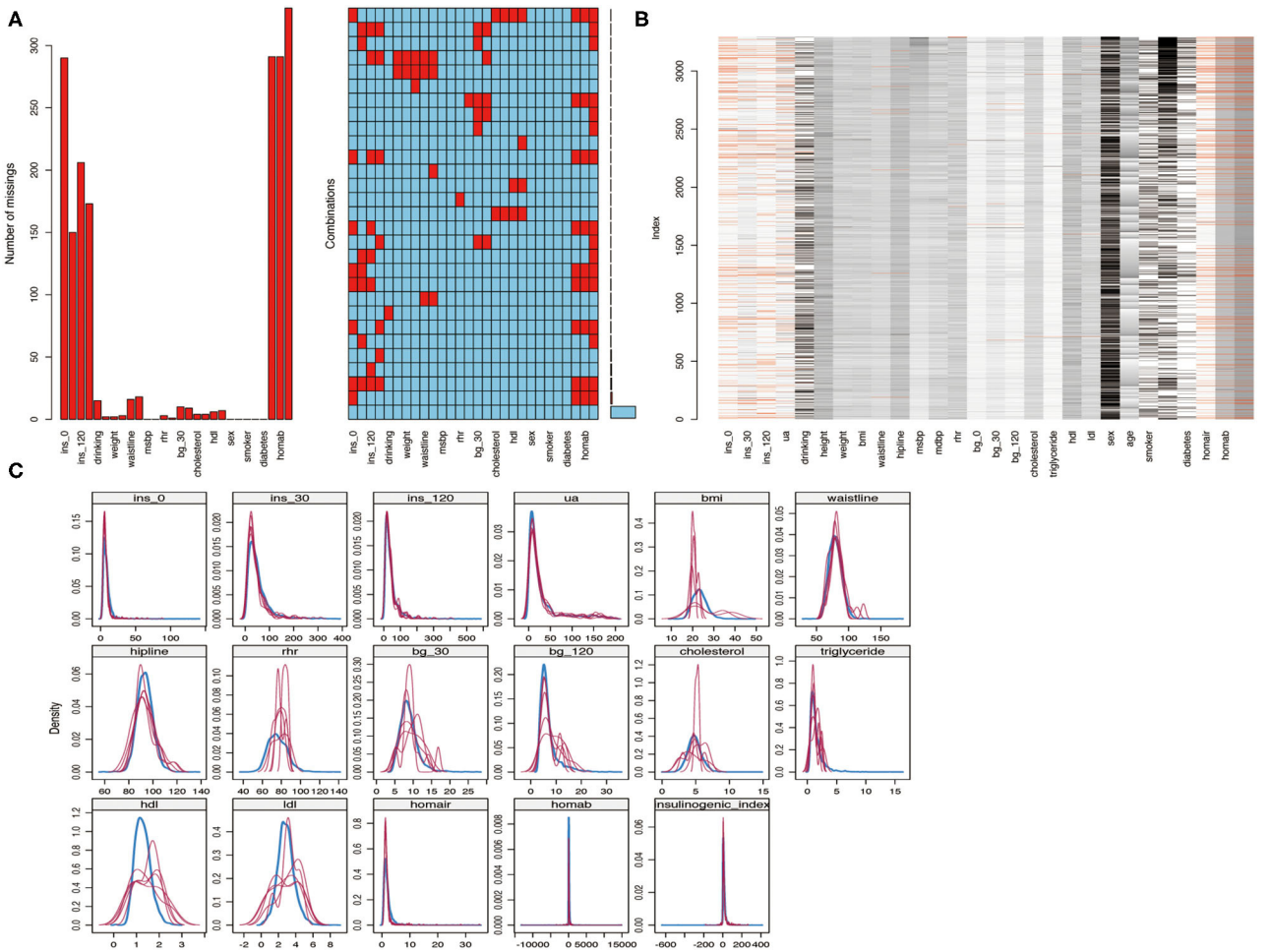

FIGURE 3 | (A,B) Analysis of missing values; **(C)** Multiple imputed data and its distribution in the original data.

Risk Model for MAU

The observed population was divided into a training set and a validation set according to a ratio of 7:3 (training set: 2,305; validation set: 989). The 2,305 cases in the training set were used to build the first predictive model, which is described in **Table 3**. Significant factors in this model (P <0.10) included SDP, DBP, Bg_0 min, TC, TG level, HDL level, gender, age, and smoking. Logistic model fitting was performed again after extracting the variables with $P < 0.10$. The second-stage model was then evaluated using a chi-square test, confusion matrix, and a calibration curve. The specificity of the model in the verification set reached as high as 0.9, with an accuracy of 0.63. The positive and negative predictive values were 0.55 and 0.65, respectively (**Figure 4A**). In the calibration curve, values of x remained close to y, indicating good calibration in both the training and validation sets (**Figures 4B,C**). Based on a $P < 0.05$, important features related to the incidence of MAU in the second-stage model included mean SBP, mean DBP, FBG, TC, TG level, HDL, gender, age, and smoking (**Figure 5A**).

Development of an MAU Risk Score

Given their significant relationship with MAU based on our analysis of the second-stage model, the following variables were used to develop the risk prediction system: mean SBP, mean DBP, FBG, TC, TGs, HDL, gender, age, and smoking. **Figure 5B** shows how total risk scores for MAU are calculated.

DISCUSSION

MAU is an early marker of diabetic kidney disease (DKD) (2), cardiovascular disease, and renal risk (1). Accounting for ∼50% of end-stage kidney disease (ESKD) cases in the developed world (16), DKD has a major effect on global healthcare costs and resources (2). Estimates indicate that the prevalence of MAU among patients with type-2 DM in the Asia-Pacific region ranges from 17.0 to 18.2%, while severe albuminuria and reduced estimated glomerular filtration rate (eGFR) are observed

TABLE 3 | Initial risk model.

	Estimate	Std. error	*P*-value
Ins_0 min (mmol/L)	−2.20e-03	1.81e-02	0.90
Ins_30 min (mmol/L)	2.65e-03	1.28e-03	0.04**
Ins_120 min (mmol/L)	−4.40e-04	1.07e-01	0.68
Drinking (Yes)	−3.02e-03	1.17e-02	0.98
Height (cm)	−1.47e-02	2.49e-02	0.55
Weight (kg)	3.01e-02	3.18e-02	0.34
BMI	−7.31e-02	8.22e-02	0.37
Waistline (cm)	7.52e-03	7.96e-03	0.34
Hipline (cm)	−8.49e-03	1.10e-02	0.44
MSDP (≥140 mmHg)	6.61e-03	3.66e-03	0.07*
MDBP (≥90 mmHg)	1.23e-02	5.74e-03	0.03*
rHR (per min)	−1.69e-03	4.48e-03	0.71
Bg_0 min (≥7 mmol/L)	1.43e-01	6.41e-02	0.03**
Bg_30 min (mmol/L)	−3.31e-02	2.74e-02	0.23
Bg_120 min (mmol/L)	3.45e-02	2.63e-02	0.19
Cholesterol (mmol/L)	−2.68e-01	1.56e-01	0.09*
Triglyceride (≥1.7 mmol/L)	1.94e-01	7.44e-02	0.01**
HDL (mmol/L)	4.65e-01	2.08e-01	0.03*
LDL (mmol/L)	2.56e-01	1.60e-01	0.11
Gender (women)	4.61e-01	1.51e-01	0.001***
Age (years)	7.47e-02	4.02e-02	0.06*
Smoking (Yes)	2.92e-01	1.43e-01	0.04**
Hypertension (Yes)	1.70e-01	1.19e-01	0.15
Diabetes (Yes)	−2.93e-01	2.14e-01	0.17
HOMA-IR	−2.39e-02	6.11e-02	0.70
HOMA-β	−6.47e-05	6.98e-05	0.35
Insulinogenic index	−3.16e-03	1.93e-03	0.10

Ins, Insulin releasing test; BMI, Body Mass Index; MSBP, mean systolic blood pressure; MDBP, mean diastolic blood pressure; rHR, resting heart rate; Bg, blood glucose; HDL, high-density lipoprotein; LDL, low-density lipoprotein; HOMA-IR, Homeostasis Model Assessment- insulin resistance; HOMA-β, Homeostasis Model Assessment of β-cell function.

*$P < 0.10$, ** $P < 0.05$, *** $P < 0.01$.*

in 2.1–14.1 and 15.3–61.6% of patients, respectively (17, 18). These statistics highlight the importance of screening, early detection, and prevention efforts to reduce the overall impact of MAU.

Given that diabetic glomerulopathy can be only be diagnosed definitively via a kidney biopsy, few studies to date have investigated methods for predicting MAU (3), making it difficult to perform a detailed analysis of MAU risk (19). DKD may be present long before the patient develops traditional indications for a kidney biopsy (20). Careful screening and prediction using the risk score developed in our study may allow for early detection of MAU without the need for a kidney biopsy.

In contrast to previous findings, DM was not identified as an independent factor influencing MAU risk in the current study. This inconsistency may be related to the low proportion of patients with DM among our participants (11.9%). In the surveyed population, elevated FBG and PBG were more prevalent than DM, suggesting that diabetes had not been identified in some patients. However, the risk associated with elevated FBG was as high as 1.11 [odds ratio (OR): 1.11, 1.05–1.19], indicating that elevated blood glucose was still an independent risk factor for MAU.

Increased intraglomerular capillary pressure, which is related to systemic blood pressure as well as pre- and post-glomerular resistance, is the most important determinant of MAU (21, 22). Previous studies have reported that blood pressure is closely associated with albuminuria in patients with hypertension and in the general population (23–25). A study conducted among the Japanese population demonstrated that SBP exhibited an independent positive correlation with MAU (21). Another Japanese study indicated that both systolic hypertension and hyperglycemia were independent risk factors for MAU, in accordance with our findings (21). Saadi et al. have also observed that SBP and DBP are significantly higher patients with MAU than in the general population (26).

One study conducted in China reported that, for each 10 mg increment in 24-h urinary microalbumin excretion within the normal range, the odds of significantly elevated TG levels increased by 41% (24). Our analysis indicated that, when compared with the normal TG range, abnormally elevated TG levels increase the risk of MAU by a factor of 1.10 [odds ratio (OR): 1.10, 1.02–1.20].

Although Ge et al. (24) observed no significant difference in gender in 24-h urinary microalbumin excretion in a study of Chinese adults, our results are in contrast to these findings. Our analysis identified gender as an important feature influencing the incidence of MAU (OR: 1.47, 1.17–1.86). In Japan, the albumin/creatinine ratio is higher in women and older adults than in men and younger individuals, respectively, but this is not true for the albumin concentration (21). Our findings also indicated that, for each 10-year increment, the odds of TG elevation significantly increased by 9% (OR: 1.09, 1.03–1.18).

Several previous studies have reported that MAU is related to smoking (27–29) and obesity (30–32), while others have noted the influence of race and region on MAU prevalence (33–35). Our study suggests that smoking is indeed an important feature affecting the prevalence of MAU (OR: 1.35, 1.02–1.77), while no such relationship was observed for obesity. However, despite appropriate calibration of the model, the low incidence of obesity among our patients may have influenced our results.

To our knowledge, the current study is the first to establish a risk score for MAU using a large sample of patients, to establish such a model using multiple imputation to account for missing data, and to utilize chi-square and logistic fitting for double-verification of model quality. Nonetheless, the study

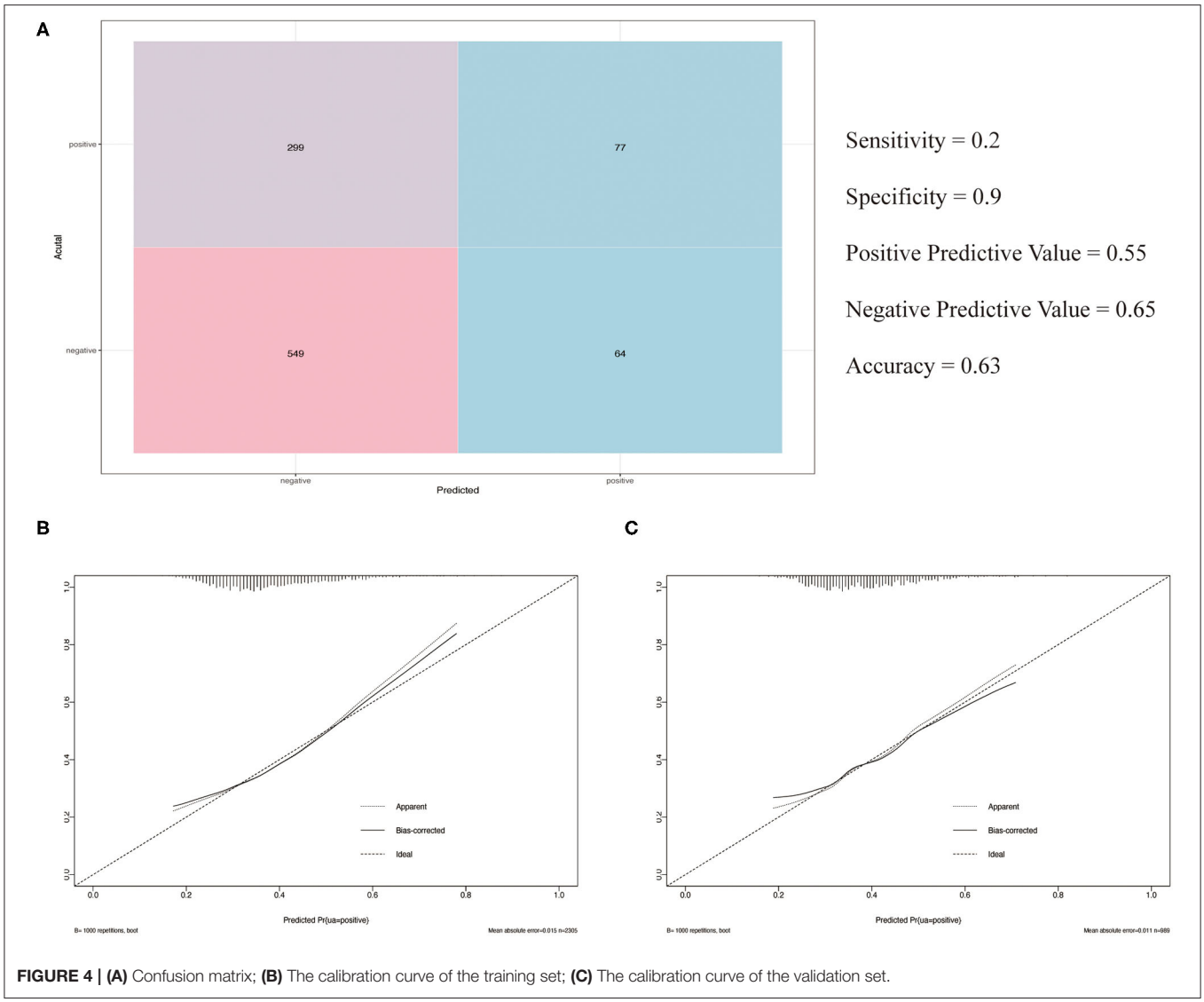

FIGURE 4 | (A) Confusion matrix; **(B)** The calibration curve of the training set; **(C)** The calibration curve of the validation set.

also had some potential limitations, including relatively limited variations in race and region. Furthermore, this was a single-center and cross-sectional study, necessitating verification of our model in multicenter studies with long-term follow-up periods.

In conclusion, based on our analysis using machine learning algorithms, we propose that comprehensive assessments of SBP, DBP, FBG, TG, gender, age, and smoking be included in the screening process for MAU. The risk score established in the present study may allow clinicians and patients to initiate early interventions that can delay or prevent the development of MAU.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Fujian Provincial Hospital Ethics Committee. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

WL and SS performed the statistical analysis and wrote the first draft of the manuscript. HH, NW, and JW reviewed, edited, critically revised the manuscript, approved the final version of the manuscript, and interpreted the data. JW and GC designed the study. GC had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

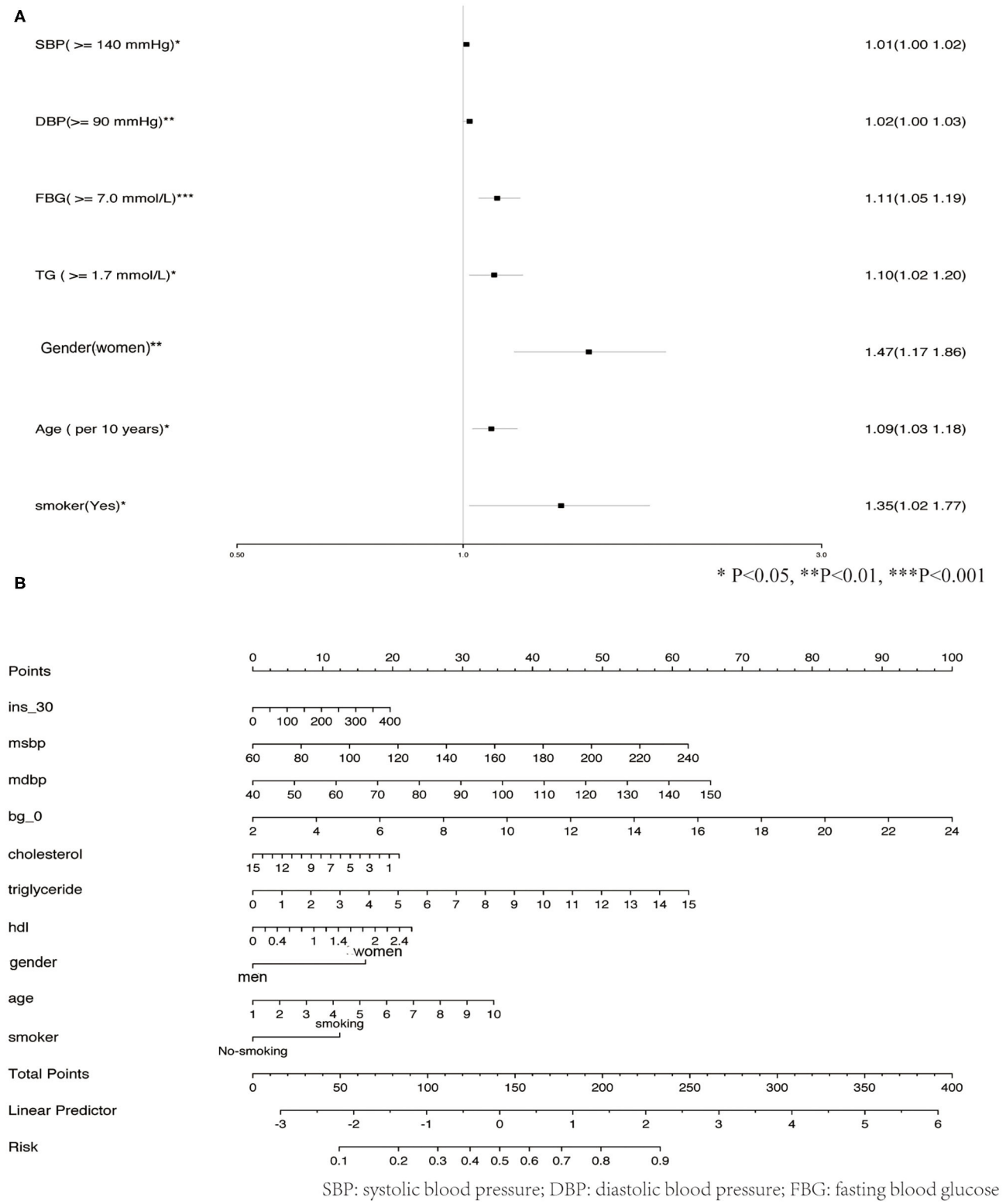

FIGURE 5 | (A) Risk model of the MAU. **(B)** Risk score to predict the risk of MAU.

All authors contributed to the article and approved the submitted version.

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Characteristics and Prognostic Value of Tertiary Lymphoid Organs in Membranous Nephropathy: A Retrospective Study

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Background: Tertiary lymphoid organs play an essential role in the inflammation of the kidney. The clinical association between TLOs and membranous nephropathy (MN) is not clear yet.

Methods: Consecutive patients with the histologically confirmed membranous nephropathy in Tongji Hospital from July 19, 2012, to September 26, 2019, were included in this study. TLOs in renal biopsy tissues were detected by periodic acid–Schiff-stained and immunohistochemistry. Logistic regression was performed to evaluate the correlations of TLOs and clinical features of patients with MN. Kaplan–Meier analysis was utilized to examine the relationship between TLOs and remission of proteinuria.

Results: A total of 442 patients with MN were included in this study, of which the average age was 46.4 years old, and 58.8% were male. Moreover, 33% of patients with MN had TLOs in this study. The median value of proteinuria among patients with MN with TLOs was 4.9 g/24 h, which was much greater than no-TLOs ones (3.2 g/24 h, $p < 0.001$). Moreover, the patients with TLOs had higher serum creatinine and lower serum albumin. The severity of clinical features among the patients with MN aggravated with the increase in the grade of TLOs. In addition, the patients who had TLOs were more likely to be positive of anti-phospholipase A2 receptor autoantibodies. Meanwhile, the patients without TLOs showed significantly higher complete remission and total remission of proteinuria.

Conclusion: In this study, we demonstrated that TLOs were common among patients with MN. Moreover, the patients with MN with TLOs showed a worse clinical manifestation and an outcome compared with the patients without TLOs.

Keywords: membranous nephropathy, tertiary lymphoid organs, anti-phospholipase A2 receptor autoantibody, proteinuria, creatinine

INTRODUCTION

Membranous nephropathy is the most common cause of adult nephrotic syndrome worldwide, representing 20–37% of cases in most series, rising up to 40% in ages over 60 years (1). Published studies showed a remarkable rising trend in the incidence of MN over the past decade (2). However, the outcome varies among patients with MN. It is reported that two-thirds of the patients with MN will experience persistent proteinuria or progress to end-stage renal disease (ESRD) over 10 years (3), and the remaining third will get a spontaneous remission without any remedies (4). Consequently, early and accurate prediction of prognosis in patients with MN is critical. Although the circulating anti-phospholipase A2 receptor (PLA-2R) is a useful serum biomarker (5), renal biopsy remains a "gold standard" in establishing the diagnosis of MN in most centers. Thus, it is of significance to defining specific pathological features associated with prognosis and disease severity of MN, as optical microscopy and immunohistochemistry can be easily assessed in clinical everyday practice.

In recent years, a growing body of literature has reported a structure of accumulated lymphoid cells, which is called tertiary lymphoid organs (TLOs), in tissues affected by non-resolving inflammation, autoimmunity, allograft rejection, and cancer (6). The prognostic value of TLOs in different diseases is contradictory. In the cancer setting, the presence of TLOs correlates with an ameliorative survival rate (7). On the other hand, in autoimmune disease, TLOs formation is classically associated with worse clinical manifestations and poor prognosis (8). A previous study showed that TLOs were observed in renal biopsy of chronic kidney disease (CKD) and related to the clinical situation of the patients with CKD (9). A recent study has reported that the number of renal TLOs was associated with poor prognosis in IgA nephropathy (10). However, the role of TLOs in MN has not been clearly elucidated. Studies suggested that TLOs formed during chronic autoimmune processes are likely to be responsible for the local generation of pathogenic autoantibody, ultimately accelerating disease progression (11). Given that MN is now considered a renal-limited autoimmune disease (12), we wondered whether renal TLOs in MN are associated with the autoantibody and disease progression.

To address the clinical significance of renal TLOs in MN, we evaluated the presence of renal TLOs in MN and analyzed whether they were associated with clinical features in a retrospective cohort. Moreover, the relationship between TLOs and anti-PLA-2R autoantibody was also explored in this study.

MATERIALS AND METHODS

Patients

In this retrospective study, 599 patients with the histologic diagnosis MN in Tongji Hospital from July 19, 2012, to September 20, 2019, were enrolled in this study. Patients with secondary membranous nephropathy were excluded ($N = 28$), including infection, tumor, other autoimmune diseases or induced by medicine. Then, we excluded the patients who had received immunosuppressant or glucocorticoids treatment before renal biopsy as well ($N = 129$). Finally, 442 patients were included in the analysis. A total of 235 patients with the data of 24-h proteinuria, including 177 patients with 24-h proteinuria $\geq$ 3.5 g, having the data of follow-up more than or equal to 1 month, were included in the analysis about the relationship of TLOs and remission of proteinuria. The range of the duration of follow-up was 1–9 months; the median follow-up duration was 5 months (**Figure 1**). The Ethical Committee of Tongji Hospital approved this study (No. TJ-IRB20210633). Due to the nature of the retrospective study, the need for informed consent from the participants of this study was waived by the Ethics Committee.

Definition of TLOs and the Examination of Anti-PLA2R Autoantibody

We firstly selected larger follicular-like structures and cellular aggregation as the candidates of TLOs by periodic acid–Schiff (PAS) stained, and then we evaluated the presence of TLOs. Immunohistochemical staining was used to confirm the existence of TLOs and the cell types in TLOs. Primary antibodies for CD3, CD4, CD8, and CD20 (Gene Tech, Shanghai) were exploited to identify different cell types. Then, 20 patients were randomly selected by a simple random sampling technique by computer-generated samples to explore the proportion of different cells in TLOs. Moreover, we used a simple grading system to evaluate the frequency of TLOs neogenesis among the patients with MN. We measured the whole area of the cortex by slide scan imaging system (SQS-40P) (Teksqray, Shenzhen). Then, the number of TLOs was normalized by the unit cortical area. Grade 1 represents without TLOs at the biopsy of kidney tissue. In the patients with TLOs, the median value of TLOs was 2.89 TLOs/10-mm^2 cortical area. Thus, we chose this value as the boundary value between Grade 2 and Grade 3. Grade 2 represents $\leq$ 2.89 TLOs/10-mm^2 cortical area, and Grade 3 represents $>$ 2.89 TLOs/10-mm^2 cortical area.

The serum anti-PLA2R autoantibody was measured by enzyme-linked immunosorbent assay in-house. An anti-PLA2R autoantibody level $\geq$14 U/ml was defined as a positive result among the patients with MN.

Definition of Remission

The total 24-h excretion was used to examine proteinuria. Serum albumin (ALB) is given in grams per liter. The patients with at least 50% reduction of proteinuria from the time of inclusion with proteinuria of $<$3.5 g/24 h, along with an improvement of serum ALB and a stable status of serum creatinine (Scr) were defined as partly remission (PR) of proteinuria. The Patients with proteinuria $<$0.5 g/24 h along with a normal range of serum ALB and a normal range of Scr were defined as complete remission (CR). The patients who achieved CR or PR were defined as total remission (TR). In addition, the patients who achieved neither CR nor PR were defined as non-remission.

Covariables

Adjusted variables were chosen on the basis of previous findings. It is reported that hypertension, proteinuria (g/24 h), serum albumin, serum creatinine, total cholesterol, and triglyceride were associated with anti-PLA2R (13, 14). As for the statistical

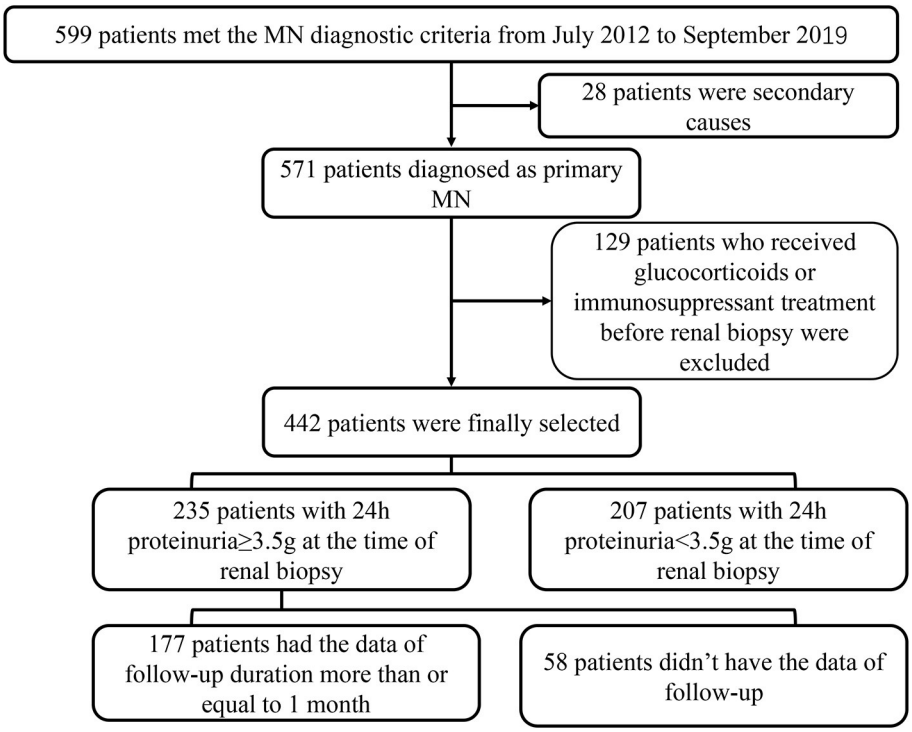

FIGURE 1 | The screening process of the patients with MN in this study. MN, membranous nephropathy.

analysis of this study, we consulted a statistician and referred to other articles. Finally, we decided to group the continuous variables. Age was grouped as 18–39, 40–59, and ≥ 60 years (15). Serum albumin was grouped as < 35 and ≥ 35 g/L (16). Serum creatinine (Scr) was divided into the elevated Scr group (> 104 umol/L for men, > 84 umol/L for women; the thresholds were given by our laboratory) and the normal group. Proteinuria was grouped as ≥ 3.5 g/24 h and <3.5 g/24 h. Total cholesterol was grouped as ≥ 6.2 and < 6.2 mmol/L; triglyceride was grouped as ≥ 2.3 and < 2.3 mmol/L (17).

Calculations and Statistics

SPSS 26.0 software (SPSS, Inc., Chicago, IL) was used to perform the statistical analyses in this study. Normal distribution tests were used for all continuous variables. The group difference of non-normal distribution was tested by non-parametric statistics. The group differences of normal distribution variables were examined by the analysis of variance. Chi-squared tests were used to perform the rate comparisons between different groups. Three logistic regression models were used to explore the relationship between the production of anti-PLA-2R autoantibody and the presence of TLOs. There was no adjustment in Model 1, Model 2 adjusted sex and age, and Model 3 further adjusted hypertension, serum ALB, Scr, proteinuria, TC, and TG. Kaplan–Meier (K-M) analysis and log-rank test were used to explore the relationship between the existence of TLOs and the remission of proteinuria, which were performed by Prism 6.0. Two-sided statistical tests were performed in this study.

RESULTS

Clinical Baseline Characteristics

Table 1 shows the clinical baseline characteristics of 442 patients with MN. The mean age of these patients was 46.4 ± 12.3 years old, and 58.8% of them were male. The initial proteinuria was 3.7 g/24 h (interquartile range, 1.9–6.1). Most of them (73.3%) were treated with immunosuppressive agents.

We found that 34.2% of the patients with MN have TLOs. The immunohistochemical staining showed that CD3+, CD8+, CD4+, and CD20+ cells were contained in renal TLOs (**Figure 2**). CD4+, CD8+, and CD20+ cells make up 34.9, 18.5, and 31.2% of all cells in TLOs, respectively, based on a subset of 20 patients randomly. We found that T cells and B cells were predominant and intermingled throughout the TLOs. Moreover, we applied the immunohistochemical marker peripheral node address in (PNAd), and there seems no HEV formation in the TLOs.

Association of Renal TLOs and Clinical Parameters

Compared with no-TLO ones, patients with TLOs were more likely to be older and male. Hypertension happened more often among the patients with MN with TLOs compared to those without TLOs, while there was no difference in the prevalence of diabetes between the two groups. In addition, there was no significant difference in treatment between these two groups (**Table 1**).

TABLE 1 | Clinical baseline characteristics of the patients with MN at the time of getting the renal biopsy.

	Total	Biopsy without TLO, *N* = 291	Biopsy with TLO, *N* = 151	*P*-value
Age,year (*N* = 442)	46.4 ± 12.3	44.7 ± 12.4	49.6 ± 11.3	0.001
Male, *N* (%)	260 (58.8)	153 (52.6)	107 (70.9)	<0.001
Hypertension, *N* (%)	109 (24.7)	56 (19.2)	53 (35.1)	0.001
Diabetes, *N* (%)	38 (8.6)	23 (7.9)	15 (9.9)	0.478
Nephrotic syndrome, *N* (%)	182 (41.2)	106 (36.4)	79 (52.3)	<0.001
Serum creatinine, μmol/L (*N* = 442)	72.0 (58.0–87.8)	67.0 (55.0-81.5)	82.0 (68.0–100.0)	<0.001
Serum albumin, g/L (*N* = 442)	27.5 (22.3–33.2)	29.4 (23.2–34.8)	25.6 (21.2–30.8)	0.001
Uric acid, μmol/L (*N* = 442)	355.8 (298.2–420.5)	349.6 (296.3–408.9)	376.0 (302.0–438.4)	0.115
TC, mmol/L (*N* = 441)	6.6 (5.1–8.2)	6.4 (5.0–7.9)	6.9 (5.4–8.4)	0.028
TG, mmol/L (*N* = 398)	2.3 (1.5–3.6)	2.2 (1.4–3.6)	2.5 (1.8–3.8)	0.016
Proteinuria, g/24 h (*N* = 442)	3.7 (1.9–6.1)	3.2 (1.6–5.3)	4.9 (2.8–7.7)	<0.001
24 h proteinuria>3.5 g, *N* (%)	235 (53.2)	138 (47.4)	97 (64.2)	0.001
eGFR, ml/min per 1.73 m^2	98.8 ± 22.9	104.3 ± 19.7	88.1 ± 25.1	<0.001
PLA−2R positive/*N*, (%)	141/255 (55.3)	83/175 (47.4)	58/80 (72.5)	<0.001
IgG, g/L	7.0 (5.1–9.5)	7.4 (5.3–9.8)	6.6 (4.8–9.3)	0.076
Treatment after diagnosed, *N* (%)				0.365
No immunosuppressive therapy	118 (26.7)	82 (28.2)	36 (23.8)	
Immunosuppressive therapy	324 (73.3)	209 (72.8)	115 (76.2)	

Values for categorical variables are given as count (percentage); values for continuous variables are given as median (interquartile range). TC, total cholesterol; TG, triglyceride; eGFR, estimated glomerular filtration rate; PLA–2R: phospholipase A2 receptor; MN: membranous nephropathy.

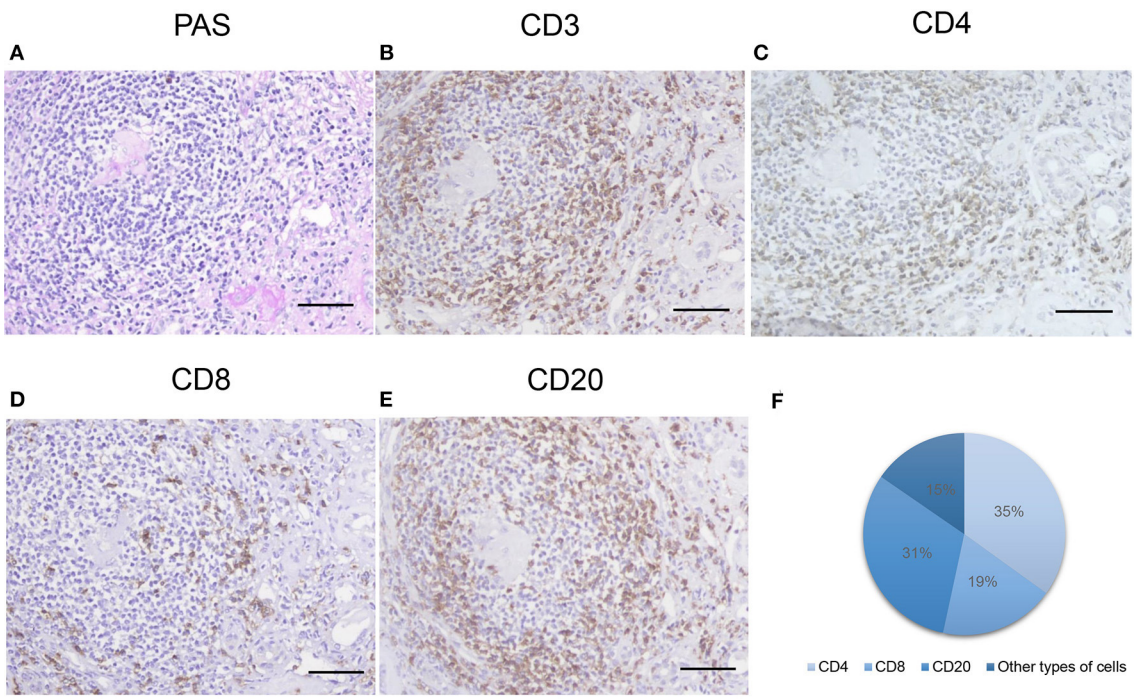

FIGURE 2 | Inflammatory cells with TLOs of the patients with MN. **(A)** Representative PAS-stained renal TLOs; immuno-histochemistry stained cells as follows: **(B)** CD3 (a T cell marker), **(C)** CD4 (a T helper cell marker), **(D)** CD8 (a cytotoxic T cell marker), **(E)** CD20 (a B lymphocyte marker). The proportions of different cell types were shown in **(F)**. TLOs, tertiary lymphoid organ; MN, membranous nephropathy; PAS, periodic acid–Schiff. Bar, 50 mm.

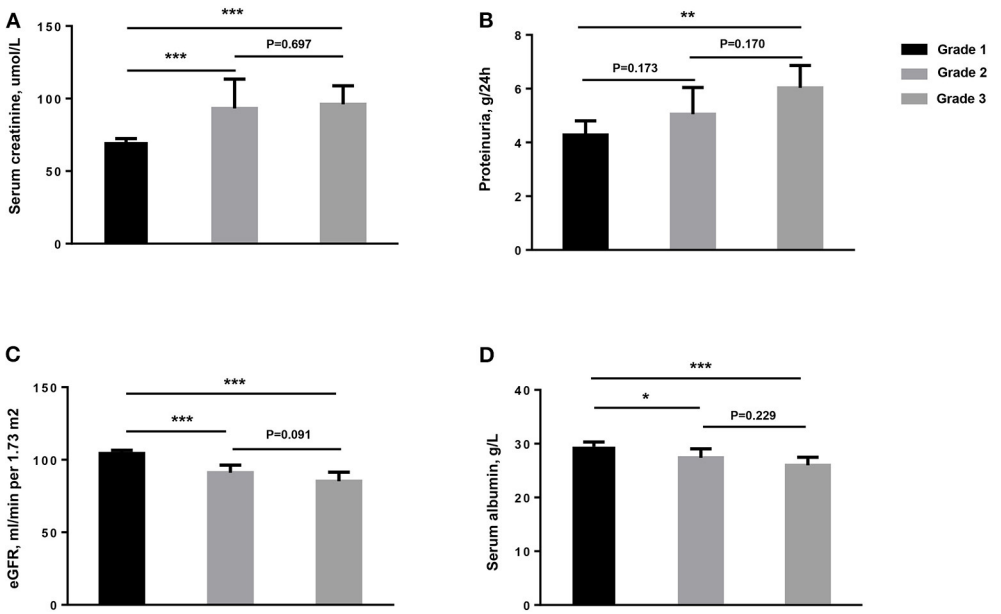

FIGURE 3 | The clinical characteristics of patients among different groups of numbers of TLOs of renal biopsy for the patients with MN. **(A)** Serum creatinine, μmol/L; **(B)** Proteinuria, g/24 h; **(C)** eGFR, ml/min per 1.73 m^2; **(D)** Serum albumin, g/L. TLOs, tertiary lymphoid organs; MN, membranous nephropathy; eGFR, estimated glomerular filtration rate. Grade 1: without TLOs. Grade 2: ≤ 2.89 TLOs/10 mm^2 cortical area. Grade 3: > 2.89 TLOs/10 mm^2 cortical area. * <0.05; ** <0.01; *** <0.001.

The median value of proteinuria among the patients with MN with TLOs was 4.9 g/24 h, which was much greater than without TLOs ones. Moreover, the patients with TLOs had higher Scr, lower serum ALB, and higher uric acid as shown in **Table 1**. We further graded the patients into three groups on the basis of the frequency of renal TLOs. The difference of proteinuria, Scr, estimated glomerular filtration rate (eGFR), and serum ALB among different grade groups is shown in **Figure 3**. We found that the more renal TLOs, the greater proteinuria and lower renal function.

Relationship of Renal TLOs and Anti-PLA-2R Autoantibody

The positive rate of anti-PLA-2R autoantibody of patients with TLOs was 72.5%, which is much higher than the patients without TLOs (**Table 1**). We further explored the relationship between TLOs and anti-PLA-2R autoantibody among the patients with MN with logistic regression, and the results are shown in **Table 2**. There showed a significant relationship between renal TLOs and anti-PLA-2R autoantibody (OR = 2.92, 95% CI: 1.65–5.19). After adjusting sex, age, hypertension, proteinuria, serum ALB, high Scr, TC, and TG, the same result was shown; the presence of renal TLOs remains independently associated with positive anti-PLA-2R autoantibody (OR = 2.27, 95% CI: 1.17–4.43).

Association of TLOs and the Remission of Proteinuria

We also studied the relationship between TLOs and remission of proteinuria among the patients with MN; 177 patients with 24-h proteinuria ≥ 3.5 g have the data of follow-up, in which 71 (40.1%) patients had renal TLOs. During follow-up, 81 (76.4%) patients without TLOs achieved remission in 9 months during follow-up (PR: 37.7%; CR: 38.7%) and 40 (56.3%) patients with TLOs (PR: 33.8%; CR: 22.5%) ($p < 0.001$). The K-M curves of proteinuria remission among the patients with MN and the remission rate of the patients with MN with and without TLOs are shown in **Figure 4**. There was a significant difference in the TR rate among the patients with TLOs and the patients without TLO ($p = 0.0121$). Then, we explored the difference in CR and PR rates among these two groups, and the CR rate showed a similar result ($p = 0.0134$) as the TR rate.

DISCUSSION

In this study, we found that the patients with MN with renal TLOs had augmented Scr and more serious proteinuria, compared to those without TLOs. Moreover, the patients with renal TLOs were more likely to be positive of anti-PLA-2R autoantibody. Notably, the presence of renal TLOs decreased the possibility of getting remission of proteinuria among the patients with MN.

To our best knowledge, this study was the first to illustrate the presence and the role of renal TLOs in MN. TLOs have been detected widely in a variety of autoimmune diseases and cancer (18, 19); however, whether renal TLOs can develop in patients with chronic kidney disease is rarely investigated. We found that renal TLOs were one of the pathological features of the patients with MN. Moreover, the presence of renal TLOs indicated poor renal condition, which is in agreement with previous studies

TABLE 2 | The relationship between PLA−2R antibody and biopsy with TLO among the patients with MN.

	PLA−2R antibody (+)/all, (%)	Model 1		Model 2		Model 3	
		OR (95%CI)	*P*-value	OR (95%CI)	*P*-value	OR (95%CI)	*P*-value
Biopsy without TLOs	83/175 (47.4)	Ref	Ref	Ref	Ref	Ref	Ref
Biopsy with TLOs	58/80 (72.5)	2.92 (1.65–5.19)	<0.001	2.69 (1.48–4.89)	0.001	2.29 (1.19–4.43)	0.013

TLO, tertiary lymphoid organ; Ref, reference.
Model 1: non-adjusting.
Model 2: adjusted sex and age (18–39 years, 40–59 years, ≥ 60 years).
Model 3: adjusted sex, age (18–39 years, 40–59 years, and ≥ 60 years), hypertension (yes vs. no), serum ALB (≤ 35 g/L vs. >35 g/L), high Scr (≥ 133 umol/L for men, ≥ 108 umol/L for women), proteinuria (g/24 h) (≥ 3.5g/24 h vs. < 3.5g/24 h), total cholesterol (≥ 6.2 mmol/L vs. < 6.2 mmol/L), and triglyceride (≥ 2.3 mmol/L vs. < 2.3 mmol/L).

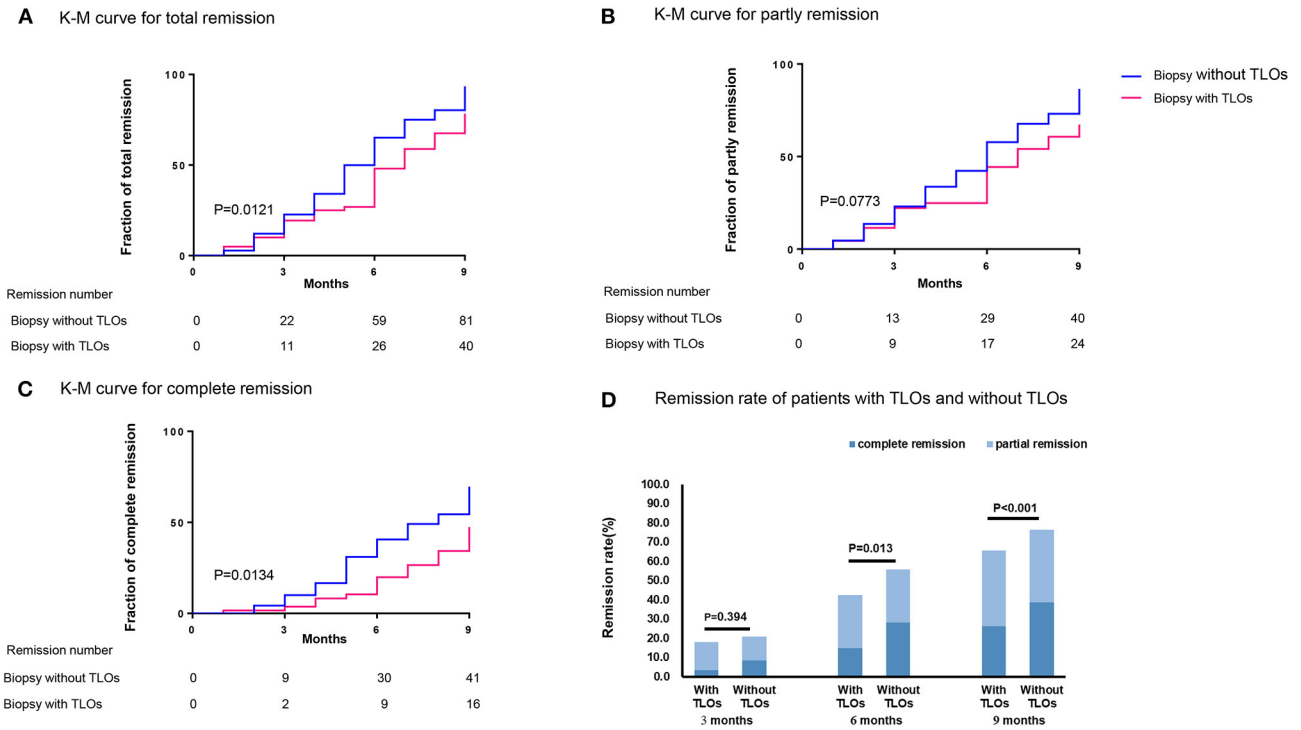

FIGURE 4 | Remission of proteinuria related to TLOs in biopsy of the patients with MN with proteinuria ≥ 3.5 g/24 h. **(A)** total remission; **(B)** partly remission; **(C)** complete remission; and **(D)** the remission rate of the patients with MN with TLOs and without TLOs in different time points. TLOs, tertiary lymphoid organs; MN, membranous nephropathy.

of patients with IgA nephropathy and lupus nephritis (20, 21). These findings suggested there is a need to consider renal TLOs as a pathological parameter in the assessment of the disease severity of MN.

Similar to TLOs detected in other diseases, renal TLOs are mostly composed of T cells and B cells (22). The well-developed TLOs provide a space in which the cooperation of inflammatory cells occurs, and thereby regulates the local immune responses of the sites of inflammation actively and influences the progression of disease (23). A previous study showed that lymphocytes in TLOs overproduce the proinflammatory cytokines under a sustained situation caused an unsolvable inflammation status in the kidney (24). Additionally, TLOs do not have capsular-like lymph nodes (25). Thus, the cells and cytokines of TLOs can expand to the renal interstitial part and occupy broad areas of parenchyma in the kidneys, which enforced the inflammation of the kidneys (9). However, a more thorough understanding of the mechanism of renal TLOs formation is still lacking, and more studies are demanded.

Our study revealed that renal TLOs might be related to the production of anti-PLA-2R autoantibody among patients with MN. A previous study showed that TLOs had a deep correlation with the generation of autoantibody, which contributed to the local pathological process within the organs (26). During the development of rheumatoid arthritis (RA), anti-citrullinated

protein antibodies' production plays an important role, and there is convincing evidence that TLOs in RA synovium produced these specificities locally (27). Moreover, TLOs' formation in the minor salivary showed an association with the presence of extractable nuclear antigen antibodies among the patients with primary Sjogren's syndrome, and these nuclear antigen antibodies indicated a worse prognosis and more severe systemic manifestation (28). In MN, PLA-2R is a kind of antigen that exists on the surface of podocytes in glomeruli (13); TLOs may also play an essential role in producing renal autoantibodies. In addition, a study revealed that the formation of TLOs was associated with an immune response against locally displayed antigens (29). Therefore, the TLOs of the patients with MN may be partly caused by the response against PLA-2R. In addition, a previous study showed that anti-PLA-2R autoantibody aggravates the proteinuria of the patients with MN (30). Meanwhile, a previous study showed that a high protein filtration rate of the glomerulus increases the inflammation of the kidney (31), which may promote the formation of TLOs in return. However, further research on a more detailed process of how TLOs and anti-PLA-2R autoantibody interacts is still warranted.

A more interesting finding of our study was that TLOs may influence the remission of proteinuria among patients with MN. A negative correlation between TLOs and clinical outcome was previously described in autoimmune disease (32). Similarly, it is reported that renal TLOs were associated with poor prognosis in IgA nephropathy and lupus nephritis (20, 33). Our results further confirmed the prognostic value of renal TLOs in MN. Moreover, As the quantification of renal TLOs is feasible under an optical microscope and by image analysis, the presence of TLOs in the kidney biopsy may be an important indicator of the management of the patients with MN. A recent murine experiment has demonstrated that inhibiting the formation of renal TLOs could reduce intrarenal inflammation and fibrosis (10). Thus, the treatment targeting renal TLOs may also contribute to preventing the disease progression in MN.

Our study also has several limitations: First, the follow-up visits were not regularly available compared to the baseline because of the retrospective design, especially for later time windows. Nevertheless, by clinical reports and records, we tried our best to get as much information as possible. Second, although most cases were well-documented, incomplete information may have existed in the medical records. Third, more features like the expression of related inflammatory cytokines did not get measured in this research.

SUMMARY

In conclusion, our study revealed that the patients with MN with renal TLOs had greater proteinuria, higher Scr, and a lower remission rate of proteinuria. Moreover, the existence of renal TLOs was related to positive anti-PLA-2R autoantibody, which provides a new sight for the production of anti-PLA-2R autoantibody. Therefore, renal TLOs may be a new clinicopathological feature to assess the disease severity and prognosis of the patients with MN.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethical Committee of Tongji Hospital. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

Z-fW and Y-cC had full access to all data in the study and take responsibility for the accuracy of the data analysis. Y-cC and S-WG developed study concepts and designs. Z-fW and Y-cC performed the statistical analyses and drafted the manuscript. Y-QL and LL provided intellectual content of critical importance to the work described. GX and S-WG obtained the funding and supervised the study. All authors contributed to the article and approved the submitted version.

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Glucocorticoids Inhibit EGFR Signaling Activation in Podocytes in Anti-GBM Crescentic Glomerulonephritis

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Glucocorticoids are commonly used to treat anti-GBM crescentic glomerulonephritis, however, the mechanism underlying its therapeutic effectiveness is not completely understood. Since podocyte EGFR/STAT3 signaling is known to mediate the development of anti-GBM glomerulonephritis, we investigated the effect of glucocorticoids on EGFR/STAT3 signaling in podocytes. We found that the levels of phosphorylated (activated) EGFR and STAT3 in podocytes were markedly elevated in anti-GBM patients without glucocorticoids treatment, but were normalized in patients with glucocorticoids treatment. In a rat model of anti-GBM glomerulonephritis, glucocorticoids treatment significantly attenuated the proteinuria, crescent formation, parietal epithelial cell (PEC) activation and proliferation, accompanied by elimination of podocyte EGFR/STAT3 signaling activation. In cultured podocytes, glucocorticoids were found to inhibit HB-EGF-induced EGFR and STAT3 activation. The conditioned medium from podocytes treated with HB-EGF in the absence but not presence of glucocorticoids was capable of activating Notch signaling (which is known to be involved in PEC proliferation and crescent formation) and enhancing proliferative activity in primary PECs, suggesting that glucocorticoids prevent podocytes from producing secreted factors that cause PEC proliferation and crescent formation. Furthermore, we found that glucocorticoids can downregulate the expression of EGFR ligands, EGF and HB-EGF, while upregulate the expression of EGFR inhibitor, Gene 33, explaining how glucocorticoids suppress EGFR signaling. Taken together, glucocorticoids exert therapeutic effect on anti-GBM crescentic glomerulonephritis through inhibiting podocyte EGFR/STAT3 signaling and the downstream pathway that leads to PEC proliferation and crescent formation.

Keywords: anti-GBM glomerulonephritis, glucocorticoids, EGFR/STAT3 signaling, Notch signaling, podocyte, parietal epithelial cell

INTRODUCTION

Anti-glomerular basement membrane (GBM) glomerulonephritis is a disease caused by autoantibodies that target collagen and are deposited on GBM. This disease manifests with rapid progression of glomerulonephritis and often reaches end-stage renal disease (ESRD) in a short time period. Treatment with glucocorticoids and other agents is effective and routinely used for the disease.

Pathologically, this disease manifests with crescent formation in glomeruli and sclerosis of glomeruli. Previous studies have shown that parietal epithelial cells (PECs) are the predominant cell type at the time when the Bowman's capsule is intact; while the Bowman's capsule is broken, several types of immune cells, including mononuclear macrophages, T lymphocytes and fibroblasts, are present as part of the crescents (1–3). Therefore, the activation and proliferation of PECs are essential for the development of crescents (4–6). Previous studies have demonstrated that podocytes are also involved in the formation of crescents (7–10). In addition to the presence of podocytes themselves in crescents, it has been suggested that podocytes that are exposed to immune deposits or inflammatory cytokines could secrete factors which promote proliferation of PECs (11–14), and several signaling pathways have been implicated in the process, including EGF signaling.

Epidermal growth factor receptor (EGFR) is a member of ErbB receptors family. Upon activation by EGF ligands (EGF, HB-EGF, etc.), EGFR is phosphorylated and subsequently activates a wide variety of intracellular signaling pathways which are essential for many cellular functions, such as proliferation, migration, differentiation, and apoptosis, etc. (15–17). In a previous study, Bollée G and colleagues showed that the phosphorylation of EGFR in podocytes by autocrine heparin-binding EGF (HB-EGF) resulted in activation of PECs and development of crescents in a mouse model of anti-GBM disease. Deletion of the *EGFR* gene in podocytes of mice alleviated crescent formation, and pharmacological blockade of EGFR was also effective even after massive formation of crescents (18). In another study, deletion of signal transducer and activator of transcription 3 (STAT3) a downstream target of activated EGFR, in podocytes was found to markedly reduce crescent formation in the mouse model of anti-GBM glomerulonephritis (19). Thus, EGFR/STAT3 signaling pathway plays a critical role in the formation of crescents.

Glucocorticoids are commonly used for the treatment of anti-GBM crescentic glomerulonephritis (20), however, the mechanism underlying the therapeutic effectiveness of glucocorticoids on the disease is elusive. In the present study, we aim to uncover the mechanisms by determining the effect of glucocorticoids on EGFR/STAT3 pathway. Our previous work has confirmed the therapeutic effectiveness of glucocorticoids on anti-GBM crescentic glomerulonephritis as shown by the improved clinical and pathological parameters and attenuated PEC proliferation and crescent formation in patients with anti-GBM nephritis (21). In the present study, we examined the expression of EGFR, and STAT3 in the glomeruli of the patients with and without glucocorticoid treatment, and further explored the molecular processes using *in vivo* and *in vitro* models.

METHODS

Human Kidney Samples

A total of 34 patients with biopsy-proven anti-GBM nephritis were recruited at our center. Kidney biopsies from patients and normal kidney tissues from nephrectomy patients were obtained. The patients were divided into two groups: group 1 treated with glucocorticoids before renal biopsy ($n = 22$) and group 2 treated without glucocorticoids ($n = 12$). Para-carcinoma kidney tissues were used as control. Patients received glucocorticoid therapy with prednisone or methylprednisolone. Samples were from Renal Biobank of National Clinical Research Center of Kidney Diseases, Jiangsu Biobank of Clinical Resources. The protocol concerning the use of the patients' samples was approved by the Human Subjects Committee of Jinling Hospital, and informed consent was obtained from all the participants.

Induction of Anti-GBM Crescentic Glomerulonephritis in Rats

Nephrotoxic serum (NTS) was prepared by immunizing New Zealand White rabbits with rat GBM as described previously (22). Wistar rats, aged 8–12 weeks and weighing 120–150 g, were sensitized with intraperitoneal injection of 0.5 mg rabbit IgG with complete Freund's adjuvant or normal saline as control. Seven days later, rats were injected with either 100 μl of NTS or normal rabbit serum alone as a control *via* the tail vein. All animal experiments were conducted following the Institute Animal Care and Use Committee of Jingling Hospital.

Animal Treatments

Wistar rats injected with NTS were randomly divided into six groups: Group 1 ($n = 5$), receiving daily intraperitoneal injections of methylprednisolone (2.5 μg/g; Pfizer) on day 1 (24 h after the injection of NTS) through 7; Group 2 ($n = 5$), daily treatment of methylprednisolone (2.5 μg/g; Pfizer) and RU486 (20 μg/g; Sigma-Aldrich) on day 1 through 7; Group 3 ($n = 5$), daily treatment of saline intraperitoneally as control; Group 4 ($n = 5$), daily intraperitoneal injections of methylprednisolone (2.5 μg/g; Pfizer) on day 7 through 13; Group 5 ($n = 5$), daily treatment of methylprednisolone (2.5 μg/g; Pfizer) and RU486 (20 μg/g; Sigma-Aldrich) on day 7 through 13; and Group 6 ($n = 5$), daily treatment of saline intraperitoneally as control. Twenty-four hour after the final treatment, all rats were sacrificed and serum samples collected. Kidney samples were processed for histological analysis, preparation of protein, total RNA, and glomeruli. Methylprednisolone is a systemic glucocorticoid that is used to treat patients with glomerulonephritis and rats of NTS model in literature.

Proteinuria and BUN Measurements

Twenty-four-hour urine samples were collected at day 1, 3, 7, 9, and 13. Twenty-four-hour proteinuria was measured using Bradford Kit (Beyotime, Shanghai) according to the manufacturer's instructions. BUN levels were measured with

the QuantiChrom Creatinine Assay Kit (BioAssay Systems, Hayward, CA).

Antibodies and Reagents

Primary antibodies, including the p-STAT3 monoclonal antibody (anti-Tyr705) (1:200 dilution, Cell Signaling Technology, #4113), the p-EGFR monoclonal antibody (anti-Tyr1068) (1:200 dilution, Cell Signaling Technology, #2234), the PAX-2 polyclonal antibody (1:200 dilution, Invitrogen, #71-60000), the CD44 monoclonal antibody (1:200 dilution, Abcam, ab16728), and the Notch1 poly polyclonal antibody (1:200, Wanlei biology, wl03097) were used to detect renal p-STAT3, p-EGFR, PECs, and activated PECs, respectively. Antibodies against EGFR (1:100 dilution, Cell Signaling Technology, ##2232) and STAT3 (1:100 dilution, Proteintech, 10253-2-AP) were used for IHC to detect total EGFR and STAT3 in kidney. Secondary antibody was the goat anti-rabbit IgG H and L (FITC) (1:200 dilution, Abcam, ab6717).

Immunohistochemistry and Immunofluorescence

Immunohistochemical staining was routinely processed. Sections were deparaffinized, rehydrated, incubated with hydrogen peroxide, and treated with microwave for antigen retrieval. Then, 10% fetal calf serum was added to block non-specific background. Subsequently, the sections were incubated with a primary antibody, followed by incubation with Envision reagent (Dako) and 3, 3′-diaminobenzidine tetrahydrochloride (DAB) as the chromogen. Then, the sections were counterstained with hematoxylin. Normal rabbit serum was used as a negative control for the primary antibody. Image pro plus version 7.0 system (Media Cybernetics, Silver Spring, USA) was used for semi-quantification of immunohistochemistry staining.

Immunofluorescence experiments in rats were performed as follows. Frozen tissue sections were fixed in Paraformaldehyde for 5 min, blocked with 5% BSA for 30 min, and incubated with goat anti-rabbit IgG H and L (FITC).

Podocyte Cell Line Culture

The cell line was the gift from Dr. Moin Saleem at Bristol University, UK (23). Podocytes were grown at 33°C and switched to and incubated at 37°C for 7–10 days. The cells were serum starved for overnight before treatment. The podocytes were treated with HB-EGF (1 ng/ml), HB-EGF (1 ng/ml) + Dexamethasone (Dex, 1 μM), respectively. Dex is a systemic glucocorticoid that is usually used for cultured cells.

Glomerular Isolation and Primary Glomerular Parietal Epithelial Cell Culture

Glomeruli from Sprague-Dawley (SD) male rats that were 6-week-old and weighed ∼200 g were isolated as previously described (24, 25). Briefly, renal cortex tissues were excised from kidney and cut into ∼1 mm^3 pieces. The tissue was then squashed through a 200-μm sieve and next passed a 70-μm sieve to retain the glomeruli on the sieve. The glomeruli were collected by gentle rinsing with cold PBS and aliquoted into dishes for culture. The percentages of capsulated glomeruli were about 20%. The isolated glomeruli were transferred onto a 6-well plate coated with type I collagen and cultured in RPMI 1640 medium supplemented with 10% FBS, 1% Pen/Strep and 1% ITS at 37°C. Glomeruli attached to the plate on day 5 of culture without any agitation. Three days later, candidate PECs were selected according to morphology, and glomeruli and other outgrowth of cells were removed from the plate. Cultured podocytes were treated with HB-EGF for 24 h, and the conditioned medium was collected and used to treat primary PECs for 24 h. The conditioned medium from cultured podocytes treated for 24 h was collected and added to the PECs. For a control that precluded any effect of HB-EGF in the conditioned medium on PECs concerning the molecules of interest, we added the same amount of HB-EGF in the control medium from the podocytes not treated with HB-EGF, and used it to treat PECs, together with the conditioned medium.

Western Blot

Cells were lysed with RIPA buffer containing protease inhibitor cocktail (Roche, Indianapolis, IN) and phosphatase inhibitor. Protein concentrations of the supernatant samples were measured with BCA protein kit (Bio-Rad). The samples were mixed with loading buffer and boiled for 5 min. Ten percent of SDS-PAGE was used for electrophoresis of the samples, and the proteins were then transferred to membrane. The membrane was incubated with blocking solution containing 5% milk in TBST solution (20 mM Tris-HCl, PH 7.14, 150 mM NaCl, 0.1% Tween-20) at room temperature for 1 h, and then incubated with primary antibodies respectively, at 4°C overnight. The membrane was washed using TBST for 5 min (3X), and then incubated with HRP-labeled secondary antibody at room temperature for 1 h. After washed, the ECL system (Millipore) was used to detect the proteins. AlphaView (ProteinSimple, USA) was used to quantify band intensities on the blots.

Real-Time Quantitative RT-PCR

Total RNA was extracted from isolated glomeruli, cultured podocytes or rat primary PECs using Mini Best Plant RNA Extraction Kit (Takara). The amount of RNA was determined using NanoDrop 1000 (Thermo Scientific, Rockford, IL). Total RNA was reverse transcribed using the PrimerScript RT Master Mix System (Takara) for first-strand cDNA synthesis. Quantitative RT-PCR was performed using Sybr Green Master Mix (Takara) and the Applied Biosystems 7900 Real-time PCR system. Ct values of the gene targets were normalized to GAPDH and 18s rRNA. The sequences of primers were shown in **Supplementary Table S1**. The fold change in the expression of target genes compared to the reference group was calculated using the $2^{-\Delta\Delta}$CT method.

Statistical Analysis

Results are presented as means ± standard errors. Differences between two groups were analyzed with Student's *t*-tests. Comparisons between three or more groups were performed using the Kruskal–Wallis ANOVA, followed by least significant difference test. $P < 0.05$ was considered statistically significant.

TABLE 1 | Characteristics of the patients with anti-GBM (21).

	GC treatment ($n = 22$)	Without GC treatment ($n = 12$)	*P*
Age (years)	41.6 ± 17.7	39.7 ± 17.7	0.77
Duration (weeks)	8 (4–12)	3 (2–7)	0.39
Gender (female: male)	0.69	1	
Pulmonary hemorrhage (%)	4.5	0	
Gross hematuria (%)	63.6	58.3	1.0
Oliguria/anuria (%)	4.5	25.0	0.12
Urinary protein (mg/24 h)	3.0 ± 3.0	3.8 ± 3.2	0.48
SCr (mg/dl)	5.43 ± 3.20	9.66 ± 5.47	0.03*
Crescents (%)	75.2 ± 27.6	84.3 ± 17.1	0.25
Cellular crescents (%)	19.1 ± 19.4	52.4 ± 32.3	0.005**
Infiltrating cells (/mm^2)	976 ± 393	1,077 ± 611	0.56

*SCr, serum creatinine; GBM, glomerular basement membrane; GC, glucocorticoid; *p < 0.05; **p < 0.01.*

RESULTS

Glucocorticoids Treatment Inhibited EGFR/STAT3 Signaling Activated in Podocytes of the Patients With Anti-GBM Nephritis

To determine the effect of glucocorticoids on EGFR/STAT3 signaling in anti-GBM nephritis, we performed immunohistochemical staining of phosphorylated EGFR and STAT3 in the renal biopsies of the patients with ($n = 22$) and without ($n = 12$) glucocorticoids treatment. The information on the patients, including renal function, proteinuria, percent crescent formation and infiltrating immune cells in kidneys was showed in **Table 1**. Other details can be found in our previous work (21). As shown in **Figure 1**, immunostaining of p-EGFR or p-STAT3 was rarely seen in glomeruli of normal human kidney. In contrast, in patients with anti-GBM nephritis p-EGFR and p-STAT3 levels were both markedly elevated in the podocytes, which typically located at the periphery of the glomerular tuft. However, the elevation of p-EGFR and p-STAT3 levels was eliminated in podocytes of patients treated with glucocorticoids.

Generation of Rat Anti-GBM Crescentic Glomerulonephritis

To prove our hypothesis that glucocorticoids suppress crescent formation and glomerulonephritis through blocking EGF signaling in podocytes, we generated a rat model of accelerated anti-GBM nephritis. In this model, Wistar rats showed heavy proteinuria 24 h after injection of nephrotoxic serum (NTS), and the urinary protein levels continuously elevated and peaked at day 9 (**Figure 2A**). Meanwhile, the concentrations of serum creatinine in NTS-treated rats were also significantly increased at day 7 and day 14 compared to that of control rats (**Figure 2B**). Concerning the pathological changes, NTS rats showed a bright linear staining of IgG deposited on GBM on immunofluorescence as previously reported. Cellular crescents were evident at about day 7, and became diffused at day 14 when 45.9 ± 6.8% of the glomeruli were crescentic and most of the crescents occupied about 15 to 40% of the area of the glomeruli (**Figures 2C**, **3E**).

Glucocorticoids Treatment Attenuated the Progression of Anti-GBM Glomerulonephritis in Rats

We investigated the effectiveness of glucocorticoids (methylprednisolone, MP) on NTS-induced glomerulonephritis in rats following the scheme in **Figure 3A**. It was found that the progressive proteinuria of NTS-treated rats in the group, in which MP treatment started the next day (day 1) of NTS injection, was alleviated at day 3 and 7. MP was also effective even if the treatment began on day 7 when progressive proteinuria and crescent had developed (**Figures 3C,D**). Consistently, elevation of serum creatinine level of the NTS-treated rats was also attenuated significantly after consecutive administration of MP for 7 days (**Figure 3B**).

In addition to the clinical parameters, pathological changes caused by NTS were mostly prevented by MP treatment as shown by decreased percentage of crescentic glomeruli (10.4 ± 4.1% vs. 45.9 ± 6.8% prior to treatment), and decreased area of the crescents (<15% area of the glomeruli) (**Figure 3E**). We additionally found that administration of glucocorticoid receptor (GR) antagonist, RU486, markedly suppressed the therapeutic effectiveness of MP as shown by the proteinuria and crescent formation in the NTS rats that were treated with MP and RU486 for seven days, which were comparable to that of NTS rats without treatment (**Figures 3C,D**). This result indicated that MP exerted its effect through its receptor.

Glucocorticoid Treatment Suppressed PEC Activation and Proliferation in NTS Rats

The activation and proliferation of PECs is an essential event in the formation of cellualr crescents. We found that the number of PAX-2 positive cells was markedly increased in the celluar crescents, as well as along the glomeular basement membrane of the rats 14 days after injection of NTS. These PAX-2 positive cells appeared to be CD44 positive as shown by immunohistochemistry staining on consecutive tissue sections showing colocalization of PAX-2 with CD44. CD44 is known to be *de novo* expressed in activated PECs in glomeruli (26). However, the numbers of proliferated and activated PECs were significantly reduced in the NTS rats after MP treatment from day 7 through 13, compared to untreated ones. Nevertheless, there was still some weak but consistent CD44 staining in glomeruli of the rats after MP treatment (**Figures 4A,B**). On the consecutive sections for PAX-2 and CD44 staining, we also observed a small portion of cells that were CD44 positive but PAX-2 negative. These cells were likely the activated mesangial cells or macrophages.

Glucocorticiods Treatment Inhibited EGF Signaling in Podocyte of NTS Rats

As described earlier, we speculated that glucocorticoids treatment could suppress the proliferation of PECs and crescent formation

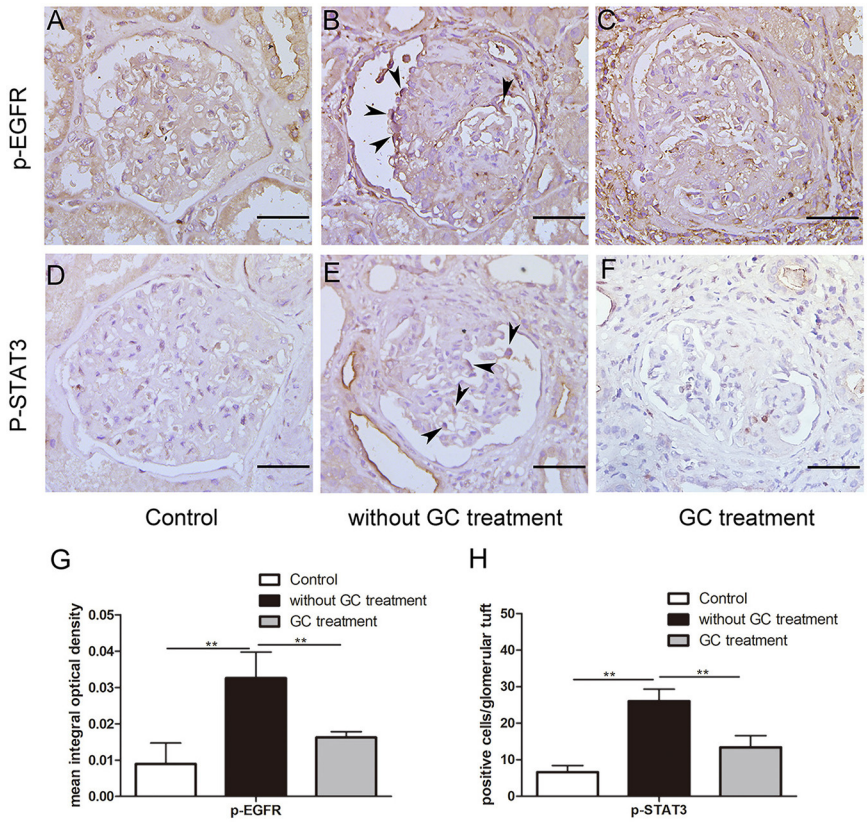

FIGURE 1 | Glucocorticoids inhibited aberrant activation of EGFR-STAT3 signaling in podocytes of patients with anti-GBM nephritis. **(A–F)** Representative immunohistochemistry staining for p-EGFR and p-STAT3 in sections of kidney biopsies from patients with anti-GBM nephritis. Twenty-two patients who received glucocorticoid (GC) treatment before renal biopsy **(C,F)** and 12 patients without GC treatment before renal biopsy **(B,E)** were analyzed. Para-carcinoma kidney tissues **(A,D)** were used as control. **(G,H)** Quantification of intensity of IHC staining in glomeruli tuft for a-f. $^{**}P < 0.01$ vs. the group without GC treatment. Scale bars, 50 μm.

by blocking EGF signaling in podocytes thus preventing podocytes to express secreted factors that promote proliferation of PECs. Previous studies have demonstrated that EGFR/STAT3 signaling pathway in podocytes is involved in the formation of crescents. Thus, we examined the activation of the components in the EGF signaling pathway by IHC staining in kidney of NTS rats. We found aberrant activation of EGFR and STAT3 in podocytes of the experimental glomerulonephritis model, as shown by the striking staining of phosphorylated EGFR and STAT3, which were not present in healthy controls and eliminated in the NTS rats with MP treatment (**Figures 4C,D**). In contrast, the total EGFR and STAT3 protein levels appeared not changed in the glomeruli (**Supplemental Figure S1**).

Glucocorticoids Treatment Reduced Notch Signaling Pathway in Podocytes and PECs of NTS Rats

Notch signaling is known to play a key role in the formation and progression of crescentic glomerulonephritis. In the present study, Notch family and their downstream transcriptional targets were examined. In kidney of control rats, Notch intracellular domain (NICD), a marker of Notch activation, was scarcely detected by IHC staining, but it was significantly upregulated in podocytes of the NTS rats (**Figures 5A,B**). Besides, quantitative reverse transcriptase–PCR (qRT–PCR) analysis with isolated glomeruli showed that expression of Notch-related genes (Notch1, Notch3, Hes1, Hey1, and HeyL) were significantly increased in glomeruli of the NTS rats compared with controls (**Figure 5C**). These results indicated that Notch signaling was activated in our experimental glomerulonephritis model. Previous studies have demonstrated that Notch signaling participates in the proliferation of PECs. Consistently, we observed NICD1 protein in PECs of the NTS rats (**Figure 5A**). Meanwhile, MP was found to prevent activation of Notch signaling as shown by the reduced NICD1 production (**Figures 5A,B**) and Notch target genes transcription (**Figure 5C**); as expected, GR antagonist RU486 suppressed these effects of MP (**Figures 5A–C**).

Glucocorticoids Treatment Suppressed Activation of EGF and Notch Signaling Induced by HB-EGF in Cultured Podocytes

To investigate the mechanism of how glucocorticoids inhibit EGF and Notch signaling, we performed *in vitro* studies using cultured podocytes. We treated podocytes with HB-EGF and

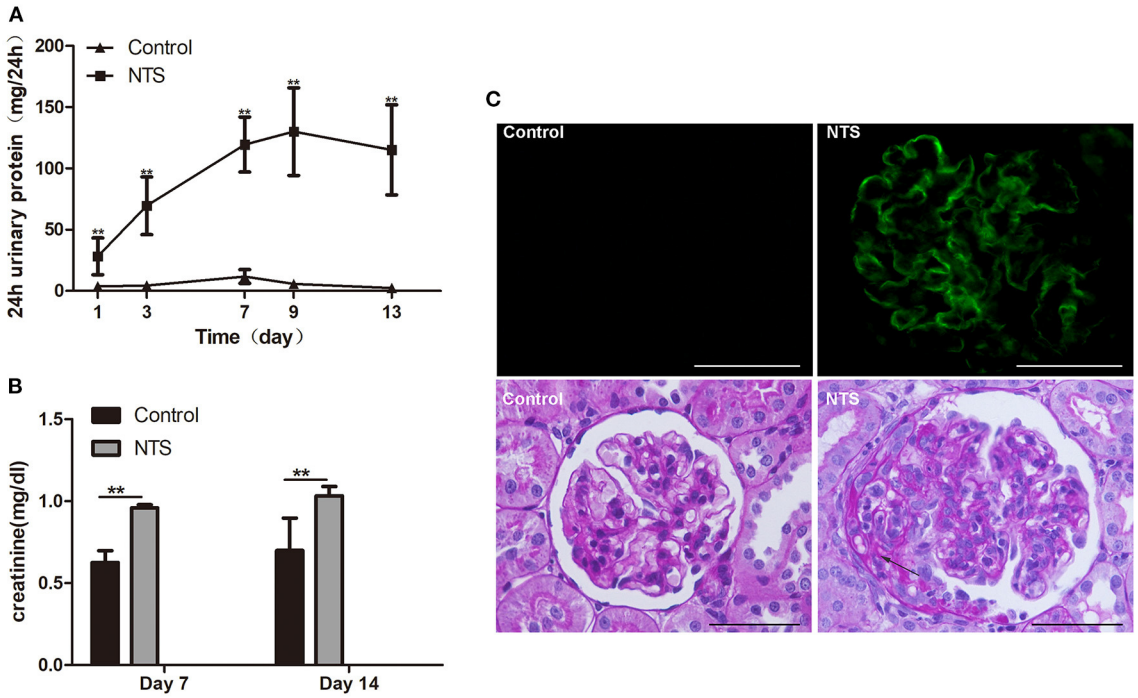

FIGURE 2 | Clinicopathologically characteristics of rat model of anti-GBM nephritis. **(A)** 24-h urinary protein levels in rats after NTS injection and control ($n = 5$ per group). **(B)** Serum creatinine concentration of NTS and control rats at day 7 and day 14 ($n = 5$ per group). **(C)** Immunofluorescence staining of goat anti-rabbit IgG in kidney of NTS rats, showing strong linear deposition of IgG along GBM; and Periodic acid–Schiff staining of kidney sections at day 14, showing crescent formation (arrows) in glomeruli of NTS rats. $^{**}P < 0.01$; Scale bars, 50 μm.

observed upregulation of p-EGFR and p-STAT3. We also observed HB-EGF-induced upregulation of p-EGFR and p-STAT3 was prevented by Dex (**Figure 6A**). However, the total protein levels of both EGFR and STAT3 in the cells were not changed (**Supplementary Figure S2**).

Notch signaling is activated in podocytes of glomerular diseases (27), and Notch3 has been further shown to be activated and promote crescent formation in glomerulonephritis in animal (28), We therefore examined Notch3 signaling in cultured podocytes treated with HB-EGF, and found it was activated as indicated by increased Notch3 intracellular domain (NICD3) (**Figure 6B**). We also found that the mRNA levels of key components of Notch signaling, including Notch1, Notch3, Hes1 and Hey1, was significantly increased in podocytes treated with HB-EGF, compared to controls (**Figure 6C**). However, the upregulation of the Notch signaling components was abolished by Dex (**Figures 6B,C**).

Conditioned Medium From HB-EGF-Treated Podocytes Activated Notch Signaling and Enhanced Proliferative Activity in Primary PECs

According to recent studies, the most important role for podocytes in the formation of cellular crescent is to secrete soluble factors that act on PECs to promote their proliferation, other than dedifferentiate and proliferate to contribute to the crescents, under stimulation of immunocomplex or inflammatory factors. Based on the results described above, we speculated EGF signaling may mediate the expression of the secreted factors in podocytes. To prove this hypothesis, we treated podocytes with HB-EGF and used the medium to treat PECs. As shown in **Figure 7A**, we established successfully the culture of primary PECs derived from isolated rat glomeruli (capsulated). The cells were treated with conditioned medium from cultured podocytes treated with HB-EGF for 24 h to test the medium for presence of factors capable of promoting proliferation of PECs. We found that mRNA expression of markers for cell proliferation (Ki-67, PCNA) and trans-differentiation (α-SMA) were significantly upregulated in the primary PECs after 24 h stimulation using the conditioned medium, compared to the controls (**Figure 7B**). Besides, Notch signaling was also activated in the cells (**Figure 7C**).

Glucocorticoids Inhibited Expression of EGFR Ligands *in vivo* and *in vitro*

To explore the mechanism underlying glucocorticoids' inhibition of EGF signaling, we tested the effect of glucocorticoids on the expression of HB-EGF, an EGFR ligand that is known to be *de novo* induced in patients with RPGN but not non crescentic glomerulonephritis (18). We observed that the mRNA expression of HB-EGF, as well as another EGFR ligand

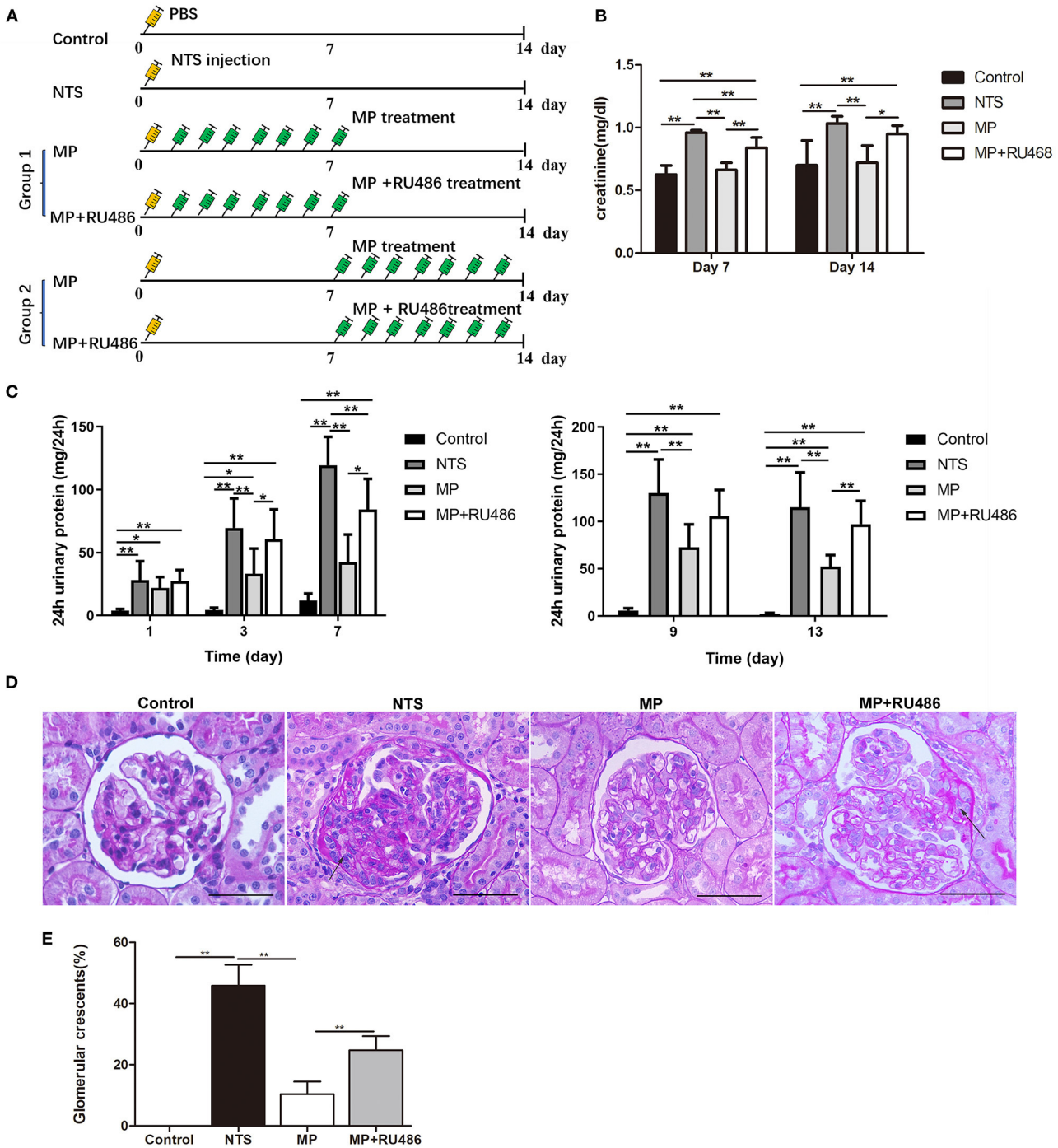

FIGURE 3 | Glucocorticoid treatment attenuated the progression of rat anti-GBM glomerulonephritis. **(A)** Schematic of experimental design. **(B)** serum creatinine concentration of rats on day 7 and 14 after NTS injection ($n = 5$ per group). **(C)** 24-h urinary protein levels of rats on day 1, 3, 7, 9, and 13 after NTS injection ($n = 5$ per group). **(D)** Periodic acid–Schiff staining of kidney from rats 14 days after NTS injection showing crescent formation (arrows) in glomeruli. **(E)** The percentage of cellular crescents in rats.$^{**}P < 0.01$, $^{*}P < 0.05$ vs. controls. Scale bars, 50 μm.

EGF, was markedly upregulated in glomeruli after induction of NTS nephritis (**Figure 8A**), which was in parallel with activation of EGFR/STAT3 signaling (**Figure 4**). MP treatment significantly inhibited the upregulation of mRNA of HB-EGF and EGF (**Figure 8A**). *In vitro*, we found that the relative mRNA expression of HB-EGF and EGF were both significantly downregulated in cultured podocytes treated with Dex. These observations demonstrated that glucocorticoids exert its effect through inhibition of EGF ligand expression (**Figure 8B**).

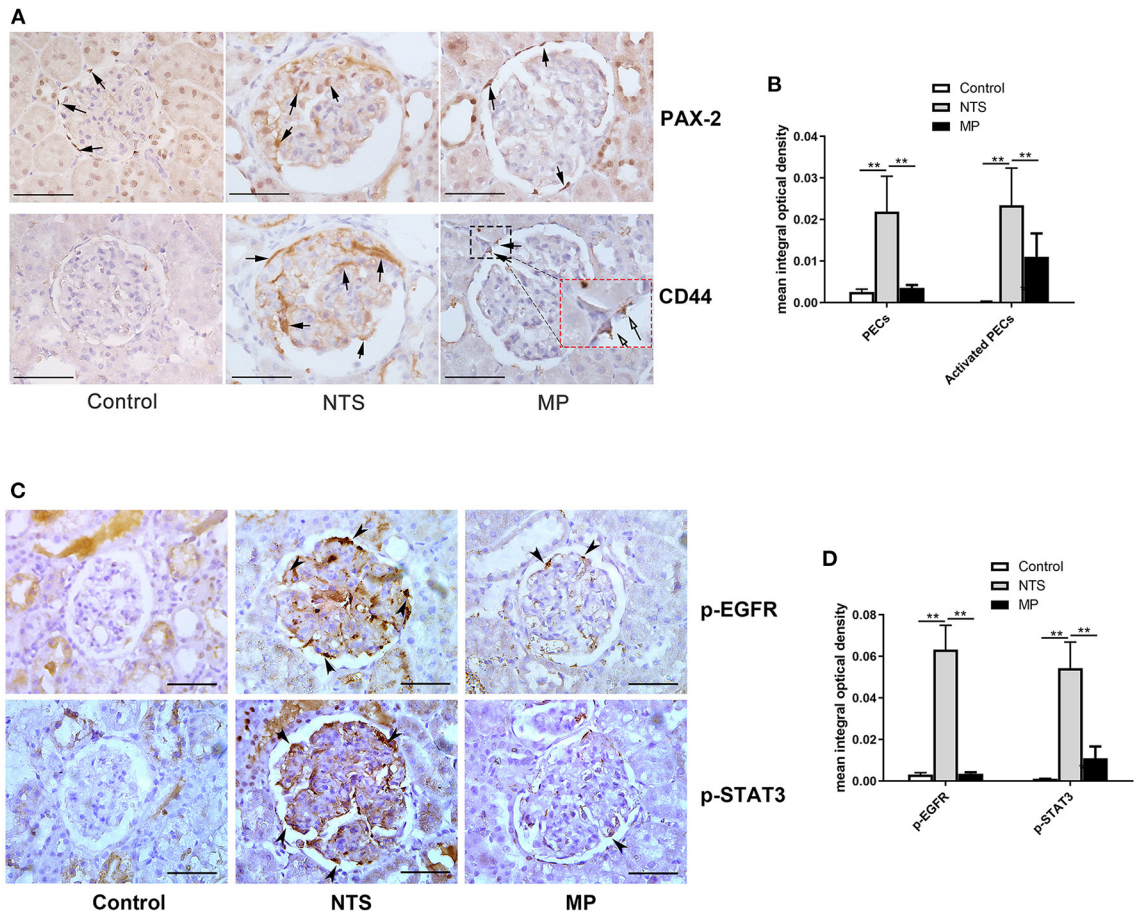

FIGURE 4 | Glucocorticoid treatment suppressed proliferation of PECs and activation of EGF signaling in podocytes of NTS rats. **(A)** Immunohistochemistry staining for PAX-2 (PECs) and CD44 (activated PECs) in sections of kidney biopsies from rats treated with NTS alone, NTS+MP, and NTS+MP+RU486, respectively ($n = 5$ per group). Quantification of staining intensity in glomeruli is shown in **(B)**; **(C)** Immunohistochemistry staining for p-EGFR (Tyr1068) and p-STAT3 (Tyr705) in sections of kidney biopsies from the corresponding rats in **(A)**. Quantification of the staining intensity for p-EGFR and p-STAT3 in glomerular tuft is shown in **(D)**. Arrows denote positive staining in the glomeruli; $^{**}P < 0.01$. Scale bars, 50 μm.

Glucocorticoids Induced Expression of EGFR Inhibitor Gene 33

EGF signaling is initiated by autophosphorylation of EGFR tyrosine kinase. Activated EGFR can be immediately targeted by the inhibitory mechanisms, including binding and inhibition by Gene 33 (ERRFI1), a negative feedback regulator of EGFR. We therefore examined the effect of glucocorticoids on Gene 33 expression in glomeruli of rats treated with NTS, and found that Gene 33 mRNA was increased by >5 times compared with control rats, suggesting an autonomous negative feedback on EGFR activation. The mRNA level of Gene 33 was further elevated after administration of MP, and the GR antagonist abolished the effect of MP (**Supplementary Figure S3A**), suggesting that glucocorticoids inhibit EGFR activation by upregulating Gene 33.

We also examined the effect of glucocorticoids on the expression of Gene 33 in cultured podocytes, and found that Gene 33 mRNA was also increased by HB-EGF but to an extent much less than that *in vivo* (**Supplementary Figure S3B**), and further increased by Dex. Consistently, Gene 33 protein was also increased by HB-EGF and further increased by Dex (**Supplementary Figure S3C**).

DISCUSSION

Anti–GBM glomerulonephritis is a form of rapidly progressive glomerulonephritis (RPGN), characterized by rapid progression, poor prognosis, and massive crescentic formation in glomeruli. As a routine and effective treatment for anti-GBM crescentic glomerulonephritis, the mechanisms underlying effectiveness of glucocorticoids have not been carefully determined. Understanding the mechanisms would likely provide improved therapeutic strategies for the disease.

Consistently with our previous studies with anti-GBM patients (21), we found that PECs and activated PECs were also increased in glomerular crescents of rat model of anti-GBM glomerulonephritis. This observation supports a key role

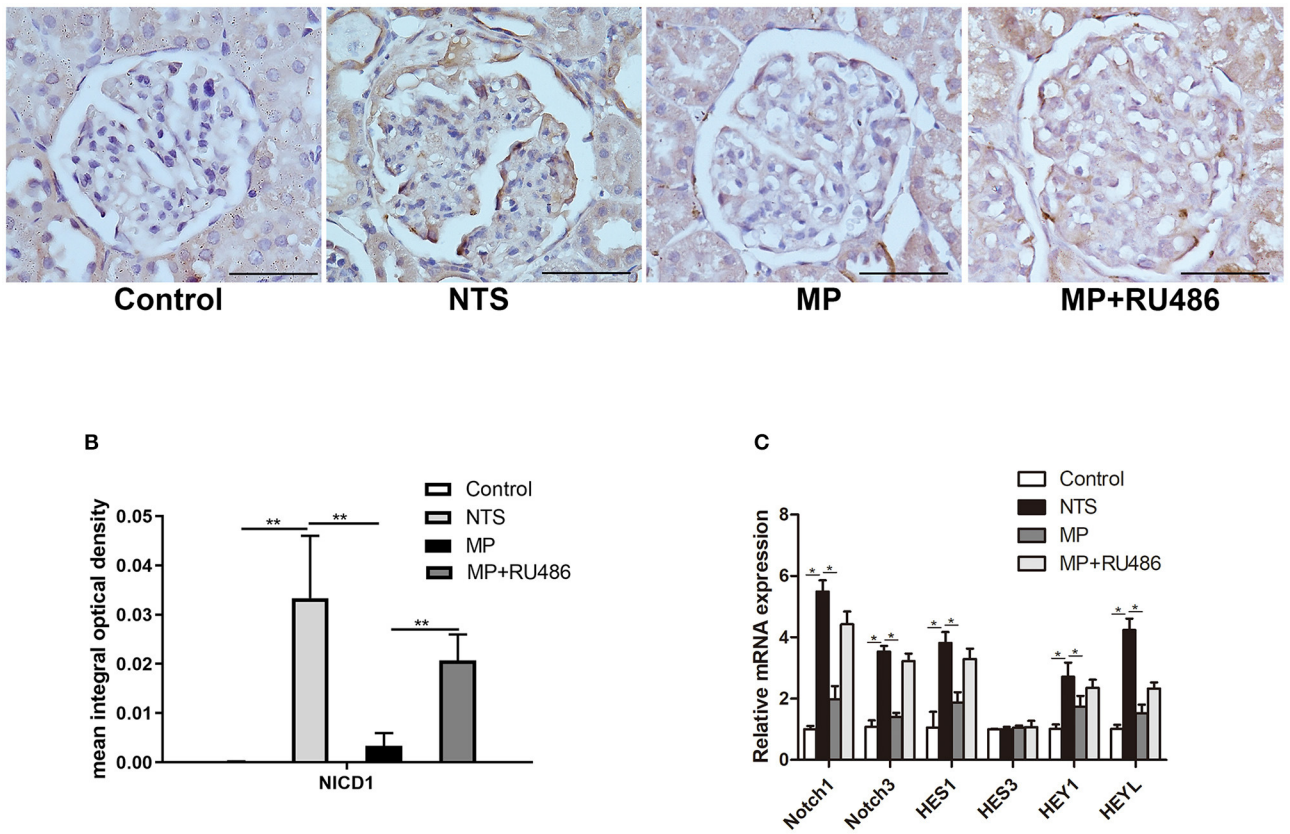

FIGURE 5 | Glucocorticoids prevented Notch signaling activation in podocytes of NTS-treated rats. **(A)** Immunohistochemistry staining of NICD1 in sections of kidney biopsies from rats treated with saline (control), NTS, NTS+MP, and NTS+MP+RU486, respectively ($n = 5$ per group). Quantification of immunostaining intensity for NICD1 in glomerular tuft is shown in **(B)**; **(C)** The relative mRNA expression of Notch pathway genes (Notch1, Notch3, Hes1, Hes3, Hey1, and HeyL) from isolated glomeruli of the rats treated as indicated on day 14 ($n = 5$ per group). $^{*}P < 0.05$, $^{**}P < 0.01$. Scale bars, 50 μm.

of PECs in crescent formation. Thus, it is crucial to elucidate which signaling triggers the activation and proliferation of PECs in order to better understand pathogenesis of the disease.

Previous studies have suggested multiple mechanisms underlying PEC activation and proliferation, including the one that stimulated podocytes secrete certain factors that promote PEC activation and proliferation (14), as suggested by the observations that EGFR or STAT3 deletion in podocytes ameliorates PEC proliferation and crescent formation (18, 19). Podocyte EGFR/STAT3 signaling may turn on the expression of secreted factors that promote PEC activation and proliferation. We did observe the activation of EGFR/STAT3 signaling in podocytes, which was accompanied by activation and proliferation of PECs, in both anti-GBM patients and rat model. In the treatment of glucocorticoids the activation of EGFR/STAT3 was abolished. This result suggests that glucocorticoids exerts therapeutic effect on the disease at least partly by preventing activation of EGFR/STAT3 pathway.

Notch signaling, as an evolutionarily conserved intercellular signaling pathway which regulates interactions between physically adjacent cells, is involved in kidney development, but its activity is rarely detected in fully developed glomeruli (29). It has been shown that Notch signaling in podocytes is required for PEC proliferation and the development of anti-GBM crescentic glomerulonephritis (28). We also observed Notch1 activation in podocytes of the NTS-treated rats, which was abolished by glucocorticoids treatment (**Figure 5**). These studies suggest a link between EGFR/STAT3 and Notch signaling in the disease. Indeed, we observed that HB-EGF was capable of inducing Notch signaling activation in cultured podocytes, indicating that Notch signaling is downstream of EGFR/STAT3. Supportively, previous studies have shown that STAT3 can directly bind to the promoter of Notch ligands, including Jagged1 and Dll1, to induce their expression (30, 31).

Le Hir et al. have demonstrated podocyte bridges between the glomerular tuft and Bowman's capsule, an early event in crescent formation (10). Given that Notch signaling is dependent on direct contact between ligand-expressing cells and receptor-expressing cells, and that STAT3 can directly bind to the promoter of Notch ligands, Jagged1 and Dll1, to induce their expression (30, 31), we may propose a new model for PEC activation and proliferation, that is, a stimulus (e.g., NTS) induces EGFR/STAT3

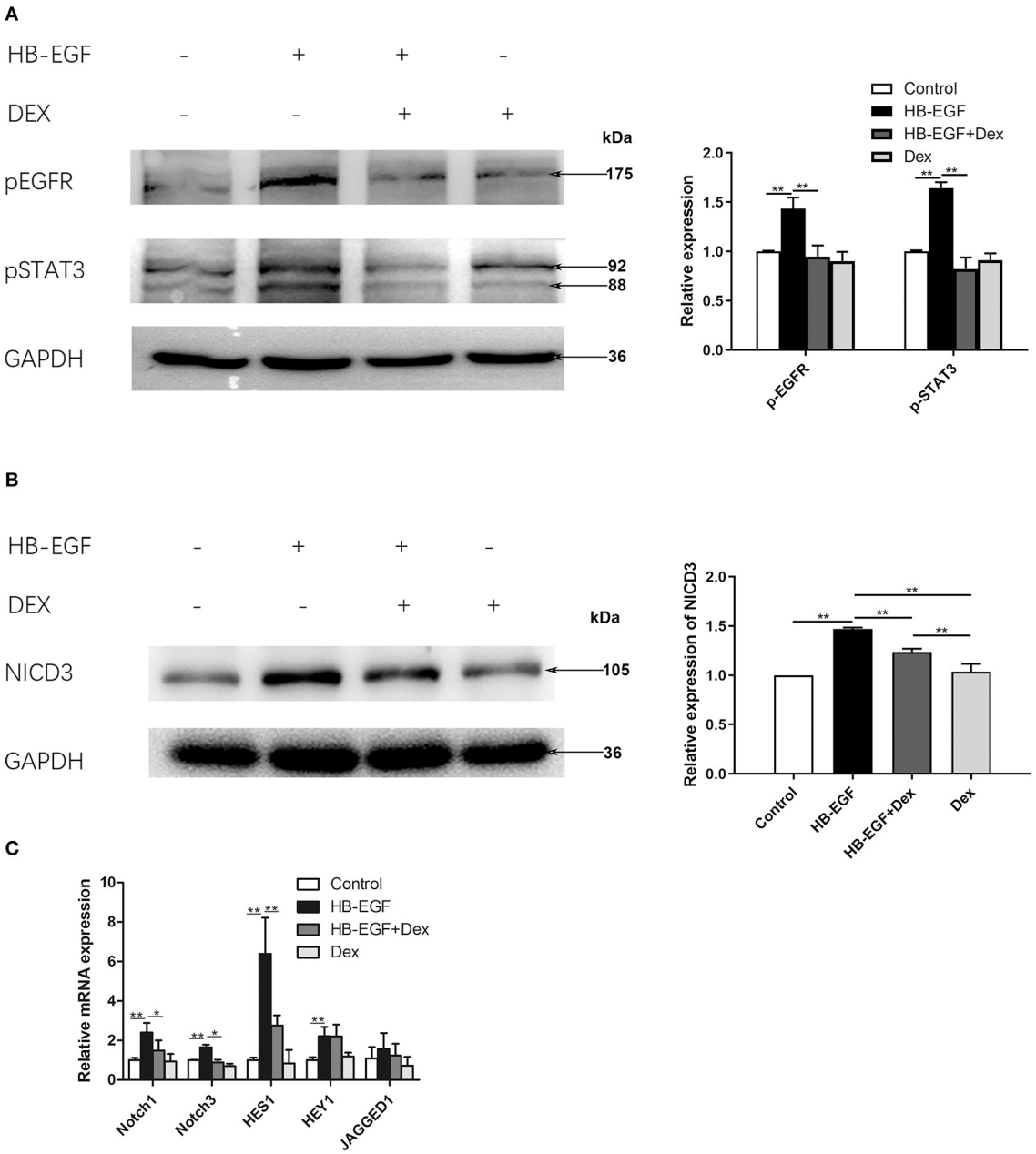

FIGURE 6 | Glucocorticoid treatment suppressed activation of EGFR, STAT3 and Notch3 in HB-EGF-treated podocytes in culture. **(A)** Immunoblotting showing that treatment of Dex prevented phosphorylation of EGFR (Tyr1068) and STAT3 (Tyr705) in HB-EGF treated podocyte (target bands are indicated by arrows) according to the samples treated for 3 h. Quantifications of the blots are shown on the right. **(B,C)** Immunoblotting **(B)** and qPCR **(C)** showing Notch signaling was induced by HB-EGF, which was prevented by Dex. Quantification of the blots is shown on the right **(B)**. The data are expressed as the mean ± SD of three independent experiments **(C)**. $^{*}P$ <0.05, $^{**}P$ <0.01 vs. mRNA levels on 0 h at baseline.

activation in podocytes; then the activated STAT3 in turn drives Jagged1/Dll1 expression; next, the Jagged1/Dll1 are presented to the Notch receptors on PECs through podocyte bridges to activate Notch signaling in PECs, resulting in PEC activation and proliferation, and eventually crescent formation. With the treatment of glucocorticoids the entire pathway is blocked from the beginning.

To prove the speculation that podocytes with activated EGF signaling could secrete factors capable of activating PECs, we treated cultured podocytes with HB-EGF and collected the conditioned medium to treat primary PECs. The conditioned medium was found to activate Notch signaling and enhance proliferative activity in the PECs (**Figure 7**). These results were consistent with previous finding that Notch signaling mediates

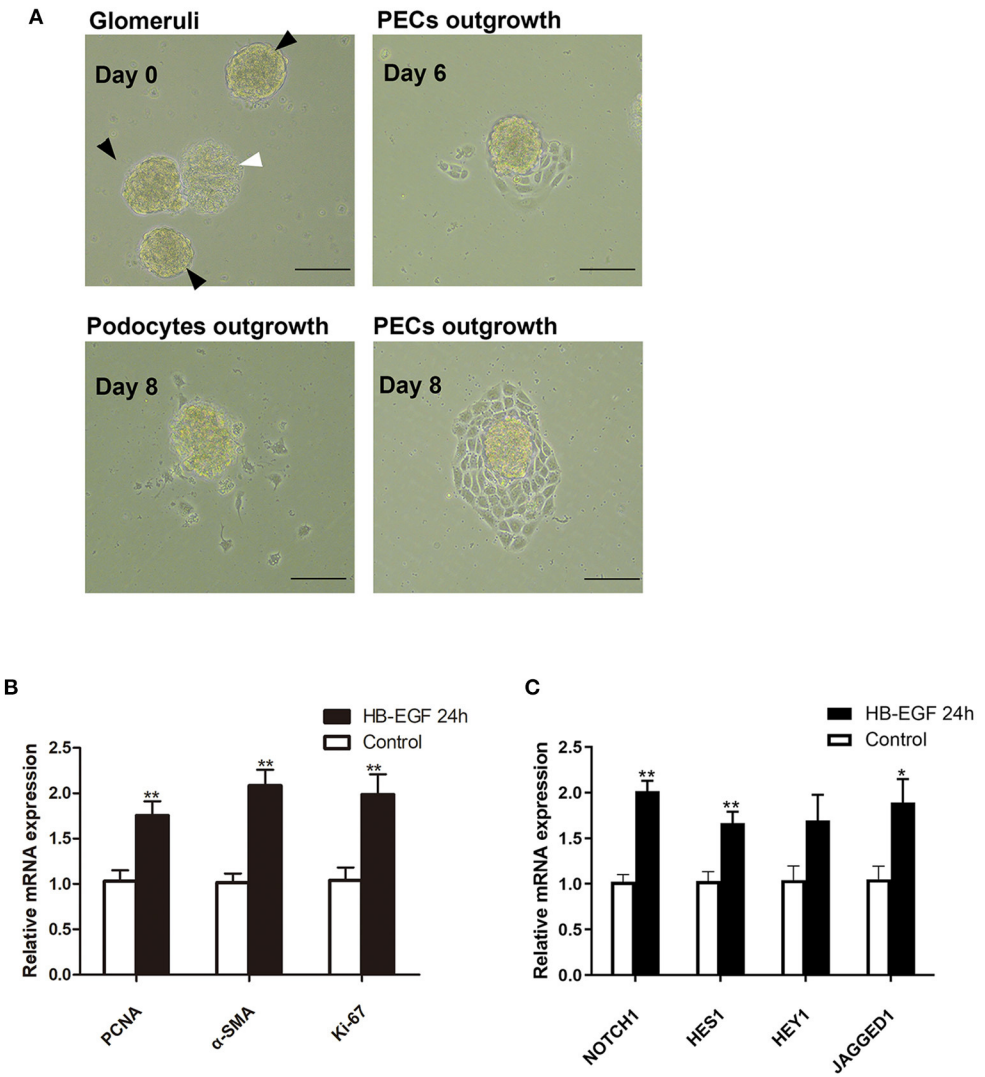

FIGURE 7 | Conditioned medium from HB-EGF-treated podocytes activated Notch signaling and enhanced proliferative activity in primary PECs. **(A)** Capsulated (black arrows) and decapsulated (white arrow) glomeruli were isolated from rat kidney. Primary PECs (irregularly shaped, uniform in size, and grown in clusters) outgrowing from capsulated glomeruli at day 7 were seen. **(B)** qRT-PCR analysis showing that the relative PCNA, α-SMA, Ki-67 mRNA expressions were significantly upregulated in the primary PECs after 24-h stimulation with conditioned medium from HB-EGF-treated podocytes **(C)** qRT-PCR analysis of Notch and downstream genes revealed a marked increase of mRNA in the same PECs as in **(B)**. All data are presented as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. primary PECs treated with control medium from untreated podocytes. Scale bars, 50 μm.

PEC activation and proliferation (28). Studies have shown that induction of Notch signaling increased the expression of mesenchymal phenotypic genes, including E-cadherin, α-SMA, vimentin, and Snail, in PECs, and promoted PEC hyperplasia and migration (32). It appears that the development of crescentic glomerulonephritis may also follow the sequence of EGFR/STAT3/Notch activation, secreted factors expression in podocytes, and Notch activation and cellular proliferation in PECs.

Glucocorticoids are known to exert therapeutic effect by genomic and various non-genomic mechanisms. The canonical genomic mechanism involves GR, thus, RU486, an antagonist of GR, can be used to determine whether an effect of glucocorticoids is through GR-dependent genomic mechanism. We showed that glucocorticoids markedly attenuated proteinuria, serum creatinine elevation, crescent formation; in addition, it effectively suppressed the activation and proliferation of PECs and other clinical and pathological worsening. Our results were consistent with other studies demonstrating podocytes are direct target of glucocorticoids (33–38). In a recent study by Zhou and colleagues, specific deletion of GR gene in podocytes was found to worsen podocyte injury after NTS treatment; however, the percentage of crescentic glomeruli was similar in the control and knockout mice (39).

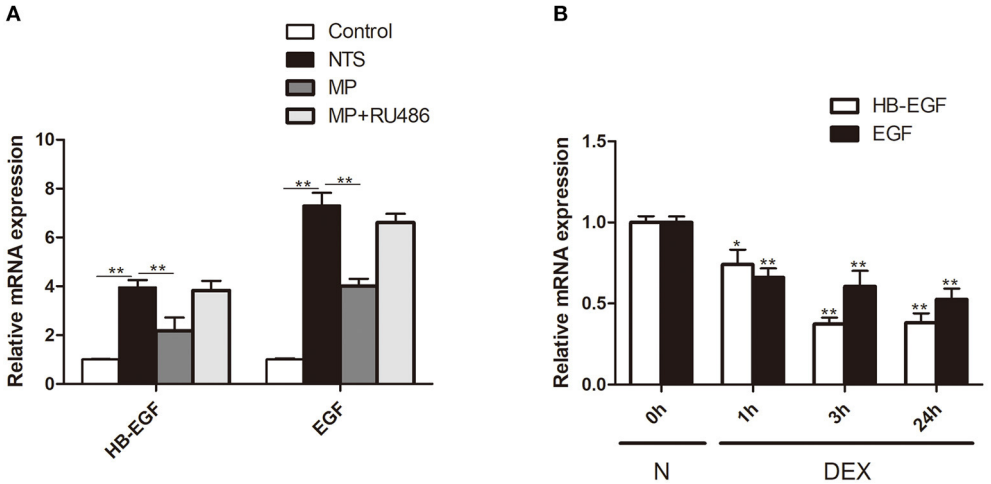

FIGURE 8 | Glucocorticoid treatment inhibited expression of EGFR ligands *in vivo* and *in vitro*. **(A)** Quantification by qRT-PCR of HB-EGF and EGF mRNA in isolated glomeruli of rats treated with saline (control), NTS, NTS+MP, NTS+MP+RU486, respectively on day 14 ($n = 5$ per group). $^{**}P < 0.01$; **(B)** qRT-PCR analysis showing the relative HB-EGF and EGF mRNA expression levels in cultured podocytes stimulated with Dex (1 μM) for 0, 1, 3, 24 h, respectively, *in vitro*. The data are expressed as the mean ± SD of three independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ vs. mRNA levels on 0 h at baseline.

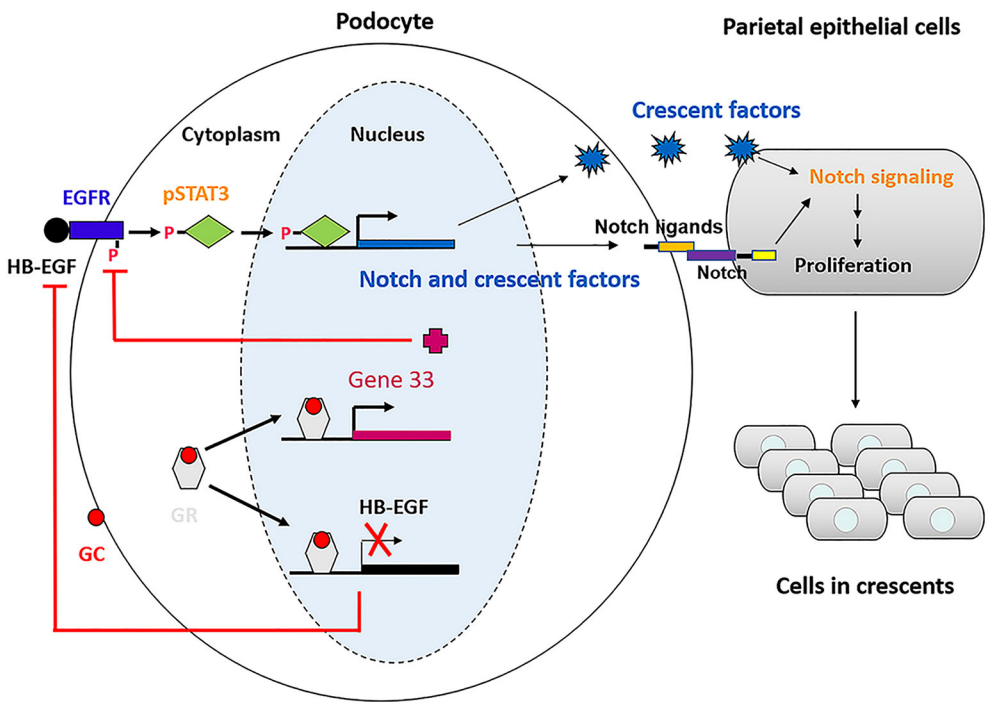

FIGURE 9 | Schematic of the mechanism underlying glucocorticoids effectiveness on anti-GBM crescentic glomerulonephritis.

To explore how glucocorticoids has inhibitory effect on EGFR activity, we performed studies in cultured podocytes. We found that glucocorticoids can inhibit expression of EGF ligands (HB-EGF, EGF), and prevent activation of EGFR, STAT3 and Notch3. These effects of glucocorticoids were abolished by RU486, suggesting that glucocorticoids inhibitory effect is through its receptor, GR, in podocytes (**Figure 8**). Apparently, expression inhibition of EGF ligands could underlie the entire effects of glucocorticoids. Furthermore, we investigated whether glucocorticoids have any effect on the expression of Gene 33, a feedback inhibitor of EGFR activation. Previous studies have shown that Gene-33 can be upregulated by EGF, HB-EGF, platelet-derived growth factor, serum, and dexamethasone (40, 41). Consistently, we also found that glucocorticoids

upregulated Gene 33 at both mRNA and protein levels in cultured podocytes (**Supplementary Figure S3**), which likely contributes to glucocorticoids-induced inhibition of EGFR and the downstream pathway. Thus, upregulation of Gene 33 may represent an additional mechanism underlying inhibitory effect of glucocorticoids on EGFR activation.

There are several limitations in the present study. Firstly, we had limited number of control groups in the animal study (**Figure 3**) and lacked the groups of MP alone, RU486 alone, and NTS+RU486. According to previous relevant studies, MP or RU486 alone does not cause overt renal changes in normal animals. However, RU486 has been shown to be protective and alleviate NTS-induced crescent formation *via* a complicate mechanism (42). We therefore did not include the NTS+RU486 group as control in order to simplify the experiments. Nevertheless, we believe that lack of the NTS+RU486 control did not affect data interpretation because the NTS+MP+RU486 group exhibited more severe injury compared with the NTS+MP group, a result that was in contrast with RU486 protective effect in the NTS-treated animals, suggesting that RU486 antagonized MP to reduce its therapeutic effectiveness.

Secondly, there was lack of co-staining of p-EGFR/pSTAT3 with podocyte markers to confirm the activation of EGFR and STAT3 specifically in podocytes. Since the cells at the periphery of a glomerulus are usually podocytes, we focused on this population of podocytes for p-EGFR and p-STAT3 localization. The results showed that p-EGFR and p-STAT3 was specifically induced in podocytes in both anti-GBM patients and NTS-treated rats, consistent with the studies by others (18, 19).

Thirdly, there might be an interference of immune cells on the results because immune cells are also responsive to glucocorticoids to reduce their injurious activity, thereby contributing to the alleviation of disease in the NTS-treated rats. Ideally, Rats with immune-cell specific GR deletion are used to exclude the influence of immune cells on the results. Nevertheless, the conclusions drawn from the present study should be still reliable because we focused on glucocorticoids effect on EGFR/STAT3 signaling which is known to contribute to glomerular crescent formation in NTS-treated animals; besides we did observe inhibitory effect of glucocorticoids on EGFR/STAT3 signaling activation. Moreover, this study also focused on the early event of crescent formation, i.e., activation of podocytes and PECs, when immune cells are blocked by intact Bowman's capsule, thus unable to enter glomeruli to play their role in crescent formation (43).

Finally, we did not perform study with podocyte-specific GR knockout mice. Zhou and colleagues made NTS model using podocyte-specific GR knockout mice and found that crescent formation was similar between the knockout and control mice (39). Unfortunately, they did not treat the mice with glucocorticoids and examined whether there was any difference in PEC phenotype and crescent formation between the knockout and control mice after NTS treatment. We would expect that glucocorticoids would have reduced therapeutic effectiveness on NTS-treated knockout mice compared with wild-type mice.

Based on our studies and previous studies by others, we propose a model illustrating the mechanism underlying therapeutic effectiveness of glucocorticoids on anti-GBM crescentic glomerulonephritis (**Figure 9**). This proposed model of mechanism may be helpful for treatment of the disease in which glucocorticoids are used.

CONCLUSION

In conclusion, aberrant activation of EGF signaling in podocytes might be a key step in crescent formation and RPGN progression. Glucocorticoids may suppress expression of EGFR ligands and upregulate expression of Gene 33, thereby preventing activation of EGFR and downstream pathway that leads to PEC activation and proliferation, and eventually alleviating anti-GBM crescentic glomerulonephritis. Further study may be required to fully prove this novel mechanism, hopefully providing new therapeutic strategies for the disease.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Human Subjects Committee of Jinling Hospital. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by the Institute Animal Care and Use Committee of Jingling Hospital.

AUTHOR CONTRIBUTIONS

ZT, SS, JZ, and XW designed the study. XW and LR performed experiments and interpreted data. QY, HS, and QT performed experiments. XW, ZT, JZ, and SS wrote the manuscript. MZ provided technical support. All the authors approved the manuscript.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.697443/full#supplementary-material

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Pregnancy in Women With Preexisting Glomerular Diseases: A Single-Center Experience

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Aim: Women with glomerular diseases are often of childbearing age. Besides lupus nephritis, data regarding pregnancy in patients with glomerular diseases are limited, posing a challenging task to attending nephrologists. This study aimed to investigate the pregnancy outcomes and the impact on the underlying glomerular disease among women followed in our institution.

Methods: A single-center retrospective cohort study of women with biopsy-proven glomerular diseases who experienced pregnancy between 2010 and 2020. We analyzed data before, during, and after gestation.

Results: A total of 22 women, 13 women with primary and 9 women with secondary glomerular diseases, were included in this study. Most patients (82%) had received immunosuppressive treatment at various times before pregnancy. All the women were in remission, either complete (62%) or partial (38%), with well-preserved renal function (82%) before conception. A total of 30 live births and 1 stillbirth were recorded; the rate of preterm delivery was 23%. Renal function and proteinuria remained stable during pregnancy. Preeclampsia was observed in 6.7% of patients and disease relapse in 6.9% of the pregnancies.

Conclusion: Pregnancy was associated with a low frequency of adverse events in women with underlying glomerular diseases, provided they have quiescent disease and preserved renal function.

Keywords: pregnancy, glomerular diseases, lupus nephritis, outcomes, maternal, fetal, relapse

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INTRODUCTION

In women with underlying kidney disease, both the effect of pregnancy on renal disease and the effect of renal disease on pregnancy may be deleterious. Women with glomerular diseases (GDs) often have proteinuria, impaired renal function, or hypertension, factors which are associated with an increased risk for adverse pregnancy outcomes (1, 2). Despite the fact that most women with underlying GD are of childbearing age, there is a paucity of data about pregnancy and GDs. The only GD with sufficient evidence published today is lupus nephritis and treatment strategies for other GD are extrapolated mostly from limited studies (3–5). In primary glomerulonephritis (GN), with the exception of immunoglobulin A nephropathy (IgAN) (6, 7), most studies report on small

numbers of patients from the older cohorts with varying rates of complications; therefore, it is difficult to draw definite conclusions about outcomes (8). In general, better outcomes have been reported for women with quiescent disease and preserved renal function at conception (4), while data on long-term follow-up before and after pregnancy are sparse. A systematic review, including pregnancy management guidelines for women with primary GD, published in 2017, has helped our understanding about the interplay between pregnancy and GD, but study heterogeneity and missing of important parameters, again preclude definite conclusions about the impact of specific factors on outcomes (9).

In this single-center study, we report the outcomes of 29 pregnancies and 30 live births in 22 women with primary and secondary GN, who were treated before and monitored during and after pregnancy in our institution. All the women had long-lasting nephritis before conception. We provide detailed description about prior treatment and disease status at conception and data about pregnancy monitoring and treatment as well as renal outcomes after delivery.

MATERIALS AND METHODS

Data Extraction

This is a retrospective single-center study. From an electronic database comprising a total of 522 patients with immune-mediated GDs in 2020, we identified 22 women with biopsy-proven primary or secondary GD who had at least one completed pregnancy. All the 29 pregnancies occurred after 2010 and were closely monitored and actively treated when necessary. All the patients had long-lasting GD before pregnancy onset and had been diagnosed, followed, and treated in our institution starting as early as in 1998. Data were collected for type of GD, GD therapy, and GD status from first diagnosis until conception. During pregnancy, an intensified, predefined visit schedule with extensive workup was introduced in all of them. Data about pregnancy management strategy and outcomes were analyzed. We were in close contact with all the treating obstetricians and data about fetal status during pregnancy, delivery, obstetrical complications, and fetal outcomes were obtained systematically and recorded in our charts. After delivery, women continued their follow-up in our center and renal outcomes until the end of 2019 were analyzed as well. All the women provided a written informed consent and this study was approved by the Local Ethics Committee.

The *aim of this study* is to highlight the impact of preconception treatment and counseling and optimized pregnancy management on outcomes in women with different subtypes of immune-mediated GDs.

Outcomes

The primary study endpoints were fetal and maternal outcomes and the secondary endpoints were maternal outcomes regarding renal parameters after delivery.

Adverse fetal outcomes included fetal death; preterm delivery, defined as delivery before completion of the 37th gestation week and classified as late preterm (34–37 weeks), moderate preterm (32–34 weeks), very preterm (28–32 weeks), and extremely preterm (<28 weeks); low birth weight (<2.5 kg) and very low birth weight (<1.5 kg) (10); neonatal intensive care unit (NICU) admission; and neonatal lupus syndrome.

Adverse maternal outcomes included nephritis flares, pre-eclamptic syndromes, worsening of renal function and/or proteinuria during pregnancy, gestational diabetes and hypertension, and obstetrical complications.

Renal parameters and status of the underlying GD were also monitored until the end of follow-up in all the women.

Renal parameters were recorded at the onset of pregnancy (baseline), at peak during pregnancy, immediately postpartum, and at the end of follow-up and were assessed as follows: renal function by use of serum creatinine at all the time points and also by estimated glomerular filtration rate (eGFR) calculated by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation before and after but not during pregnancy. Urine protein was measured in 24-h urine collection and presence of active or inactive urine sediment was examined. GD status was assessed based on these parameters and complete or partial remission or disease flare was defined according to validated criteria for each one. In patients with primary GD, complete remission was defined as a reduction of proteinuria to <0.3 g/d and normal serum creatinine; partial remission was defined as a 50% decrease of proteinuria and urine protein levels 0.3–3.5 g/d and improved or stable serum creatinine. In patients with lupus nephritis, complete remission was defined as a reduction of proteinuria to <0.5 g/d and return of serum creatinine to previous baseline value; partial remission was defined as a 50% decrease of proteinuria and urine protein levels 0.3–3.5 g/d and improved or stable serum creatinine. In antineutrophil cytoplasmic antibody (ANCA)-associated systemic vasculitis (AASV), remission was defined as the absence of microscopic hematuria and a stable or improved proteinuria and eGFR (11).

Preconception Counseling

As all the patients had long-lasting disease with regular follow-up in our institution, once they expressed the wish to conceive, they received a counseling visit performed by a nephrologist. After careful assessment of potential risks and after having discussed them in detail with the patient, the decision was in most cases supporting, in some cases postponing, and in rare cases rejecting the will for pregnancy according to the current status of patients. Patients were advised to defer pregnancy until the disease was well-controlled for at least a year [i.e., complete or partial remission of GD according to the Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, no flares or relapses within the past year] or in cases of advanced chronic kidney disease (CKD) (eGFR < 45 ml/min/1.73 m^2) (11). Almost all the decisions were taken in agreement of the physician and the patient, but in some cases the patient insisted and indeed, proceeded with pregnancy despite having been instructed to withhold.

Treatment of Underlying Nephritis Before Pregnancy

Immunosuppressive medications, duration of immunosuppressive therapy, and time from discontinuation

TABLE 1 | Demographic and baseline characteristics.

Patients	Race	GD	Age at GD diagnosis	Age at pregnancy onset*	sCr at pregnancy onset	uPR at pregnancy onset	BMI at pregnancy onset	Comorbidities
1	Caucasian	IMN	18	34 39	0.6 0.66	1.27 1.59	21 22	Hypertension
2	Caucasian	IMN	29	32 36	0.6 0.6	1.37 0.19	24 25	None
3	Caucasian	IMN	34	37	0.57	0.13	24	Hypertension
4	Caucasian	IMN	28	34	0.76	0.19	22	None
5	Caucasian	FSGS	22	32 34	0.6 0.6	0.12 0.05	26 29	None
6	Caucasian	FSGS	33	40	1.6	0.20	22	Hypertension
7	Caucasian	FSGS	14	30	1.3	0.89	32	Hypertension
8	Caucasian	FSGS	21	35	0.6	0.81	20	Hypertension
9	Caucasian	IGMN	18	30	0.6	0.91	23	None
10	Caucasian	MCD	31	33	0.6	0.16	22	None
11	Caucasian	MCD	29	39	0.59	0.04	24	None
12	Caucasian	IGAN	24	29	0.6	0.19	20	None
13	Caucasian	IGAN	16	36	2.0	1.06	20	None
14	Caucasian	LN	28	41	0.6	0.18	23	None
15	Caucasian	LN	19	32 33	0.6	0.8	20	None
16	Caucasian	LN	22	25	0.6	0.08	24	None
17	Caucasian	LN	29	34 36 38	0.6 0.7 0.6	0.10 0.05 0.16	23 23 22	None
18	Caucasian	LN	28	37	0.69	0.19	29	None
19	Caucasian	LN	14	34	1.31	0.65	20	None
20	Caucasian	LN	21	28	0.64	0.64	29	None
21	Caucasian	AAV	27	32 36	0.11 0.79	0.6 0.67	30 28	None
22	Caucasian	AAV	30	37	1.27	0.84	29	None

IMN, idiopathic membranous nephropathy; FSGS, focal segmental glomerulosclerosis; IGMN, IgM nephropathy; MCD, minimal change disease; IGAN, IgA nephropathy; LN, lupus nephritis; AAV, antineutrophil cytoplasmic antibody-associated vasculitis; GD, glomerular disease; sCr, serum creatinine; uPR, urine protein; BMI, body mass index.
**The upper number of each cell corresponds to the age of the first pregnancy.*

TABLE 2 | Diagnosis of glomerular disease ($n = 22$).

Kidney biopsy diagnosis	No. of patients ($n = 22$)	Percent of patients (%)	Median time from GD diagnosis until pregnancy (months)
Membranous nephropathy	4	18	69
Focal segmental glomerulosclerosis	4	18	114
IgM nephropathy	1	5	150
Minimal change disease	2	9	65
IgA nephropathy	2	9	153
Lupus nephritis	7	32	107
ANCA-associated vasculitis	2	9	85

until conception or supportive treatment only with renin-angiotensin-aldosterone system (RAAS) blockade or angiotensin receptor blockers (ARBs) were recorded.

Follow-Up During Pregnancy

A first visit was scheduled immediately after conception and at predefined time intervals after that: once a month until the 20th gestation week, every 15 days from the 20th to the 28th gestation weeks, and once a week from the 28th gestation week until delivery, respectively. Besides routine laboratory investigation, 24-h urine protein, office and home blood pressure measurement, weighting and clinical examination, and contact with the treating obstetrician were performed at every visit. Medical treatment during pregnancy was administered in cooperation with the obstetrician and also recorded in our charts at every visit.

In patients with lupus nephritis, extensive immunological workup including anti-dsDNA antibodies, serum complement factors C3 and C4, antiphospholipid antibodies (APAs) [anticardiolipin immunoglobulin G (IgG) and immunoglobulin M (IgM), anti-β2 glycoprotein I (β2GPI) IgG and IgM], and anti-Ro/Sjögren's syndrome-related antigen A (SSA) and anti-La/SSB antibodies was assessed at first pregnancy visit and by indication after that and systemic lupus erythematosus (SLE) disease activity

TABLE 3 | GD status and therapy from diagnosis until pregnancy. Patients with primary GD ($n = 13$).

Disease	Diagnosis of GN (year)	Pregnancy onset (year)	Time since Diagnosis (months)	Therapy	Duration of immuno-suppression (months)	Time since immuno-suppression discontinuation (months)	Duration of remission prior to pregnancy (months)	Disease status at pregancy
IMN	1997	2013 2018	192 252	RAAS inhibitors	-	-	5 65	PR
IMN	2011	2014 2017	29 65	RAAS inhibitors	-	-	24 60	CR
IMN	2016	2019	32	CYC+CS	6	22	18	CR
IMN	2012	2018	57	CYC+CS+ CSA+RTX	57	14	34	CR
FSGS	2000	2010 2012	114 138	CSA+CS	72	24 46	48 70	CR
FSGS	2006	2013	84	CSA+CS	60	13	48	PR
FSGS	1998	2014	184	CS	18	168	57	PR
FSGS	2003	2017	48	RAAS inhibitors	-	-	36	PR
IGMN	2003	2015	150	NONE	-	-	14	PR
MCD	2011	2013	24	CS	12	12	23	CR
MCD	2009	2018	106	CS	12	24	31	CR
IGAN	2008	2013	66	CYC+CS	16	48	60	CR
IGAN	1993	2013	240	CS	NA	6	6	PR

IMN, idiopathic membranous nephropathy; FSGS, focal segmental glomerulosclerosis; IGMN, IgM nephropathy; MCD, minimal change disease; IGAN, IgA nephropathy; RAAS, renin angiotensin aldosterone system; CYC, cyclophosphamide; CS, corticosteroid; CSA, cyclosporine; RTX, rituximab; GD, glomerular disease.

index (SLEDAI) was calculated. Rheumatologic consultation was considered in case of severe extrarenal manifestations.

RESULTS

We identified 29 pregnancies in 22 women, all with preexisting, biopsy-proven primary and secondary GD. Demographic characteristics and kidney biopsy diagnoses are shown in **Tables 1**, **2**. All the patients had long-lasting disease with median time from GD diagnosis until pregnancy of 103 (range 24–252) months.

Previous Treatment of the Underlying GD

In the majority of patients, 18 out of 22 (82%) patients had received immunosuppressive therapy before the onset of pregnancy. In total, 8 patients had received cyclophosphamide (CYC), 3 patients had received calcineurin inhibitors (CNIs), 4 patients had received mycophenolic acid (MPA), 4 patients had received only steroids, 4 patients had received rituximab, and 2 patients had received azathioprine. Four patients, two patients with membranous nephropathy, one patient with focal segmental glomerulosclerosis (FSGS), and one patient with IgM nephropathy, all with subnephrotic proteinuria for long, had received only RAAS blockade.

Disease Status at Presentation

At pregnancy onset, all the patients had remission of GD, either complete, 18 out of 29 (62%) or partial in the remaining 11 out of 29 (38%) pregnancies, respectively. None of the patients was on immunosuppression (IS) at conception. Median time from IS discontinuation until conception was 22 (0–168) months. RAAS inhibitors were withdrawn in all the patients as soon as they started planning to conceive or immediately upon notification in case of an unplanned pregnancy. GD status and therapy from diagnosis until pregnancy are given in **Tables 3**, **4**.

Ten pregnancies were recorded in 7 patients with SLE nephritis. At onset, 4 and 5 patients had only slightly elevated dsDNA titers and low complement (C3 and/or C4) levels, respectively. One patient was positive for anti-Ro/SSA Abs and one patient was positive for APAs. Median SLEDAI index was 2.0.

Median age of patients at conception was 34 years (range 25–41 years) with 10 out of 29 (35%) patients being over 35 years. Median body mass index (BMI) was 23.2 kg/m^2 (range 19.9–32 kg/m^2), 7 out of 29 (24%) patients were overweight and one patient was obese. Most women had a natural pregnancy, while 4 out of 22 women conceived after assisted reproductive technology (ART), which comprised ovulation induction (OI) and *in vitro* fertilization (IVF).

A total of 29 pregnancies were recorded in 22 women, resulting in 30 live births: two out of 29 were twins, both in women after ART, while 6 patients had more than one pregnancy; five had 2 and one even three consecutive pregnancies.

Pregnancy outcomes are shown in **Tables 5**, **6**.

Fetal Outcomes

A total of 29 pregnancies resulted in 30 live births. There was no neonatal death, while there was one stillbirth in the 23rd week due to maternal chorioamnionitis. Median gestation time was 37

TABLE 4 | GD status and therapy from diagnosis until pregnancy. Patients with secondary GD ($n = 9$).

Disease	Diagnosis of GN (year)	Pregnancy onset (year)	Time since diagnosis (months)	Therapy	Duration of immuno-suppression (months)	Time since immuno-suppression Discontinuation (months)	Duration of remission prior to pregnancy (months)	Disease Status at Pregancy
LN	1998	2011	156	CYC+MPA+ CS+AZA+ RTX	156	0.8	48	CR
LN	1999	2012 2013	156	MPA+CS	60	0.8 3	20 7	PR PR
LN	2012	2015	46	MPA+CS	45	0	37	CR
LN	2009	2014 2016 2018	62 91 109	MPA+CS +RTX	59	3 34 48	56 90 103	CR CR CR
LN	2007	2016	106	MPA+CS	88	16	93	CR
LN	1998	2018	243	PLEX+CYC+ MPA+CS+ AZA+RTX	192	46	60	PR
LN	2011	2018	80	CYC+MPA+CS	53	27	76	CR
AAV	2010	2015 2019	60 103	CYC+MPA+ CS	48	12 57	54 96	CR CR
AAV	2012	2019	85	CYC+MPA+CS	30	55	72	CR

LN, lupus nephritis; AA, antineutrophil cytoplasmic antibody-associated vasculitis; CYC, cyclophosphamide; CS, corticosteroid; MPA, mycophenolic acid; AZA, azathioprine; RTX, rituximab; PLEX, plasma exchange; GD, glomerular disease.

weeks (range 23–39 weeks) with a preterm delivery rate of 21% (6 out of 30) for premature delivery before the 37th week and 3.4% (1 out of 30) for delivery before the 34th week, respectively.

Out of the 30 live newborns, 5 (16.6%) had low (<2.5 kg) and 4 (13.3%) had very low (<2 kg) birth weight. Of the 30 neonates, 6 (20%) were admitted to the neonatal intensive care unit (NICU).

Out of 10 pregnancies and 11 newborns in 7 patients with lupus nephritis, there was no case of neonatal lupus syndrome.

TABLE 5 | Fetal/neonatal outcomes.

	No.	Percentage
Fetal Loss		
Neonatal death	none	0
Stillbirth at >20 weeks	1	3.4
Preterm delivery		
Late (34–37 weeks)	6	20
Moderate (32–34 weeks)	1	3.4
Low birth weight		
2.0–2.5 kg	5	16.6
1.5–2.0 kg	4	13.3
ICU admissions	6	20

Percentage per total number of newborns (n = 30).

Maternal Outcomes

There was no maternal death nor major complication as thrombosis, stroke, or sepsis in our cohort. Two pre-eclamptic syndromes occurred in 29 pregnancies (6.7%): one case of preeclampsia in week 36 in a patient with membranous nephropathy and one case of HELLP syndrome in week 32 in a patient with SLE nephritis. Both resulted in successful emergent delivery without major adverse outcome, neither for the neonates nor for the mothers. Two more patients flared during pregnancy: one with SLE nephritis in the 17th week of gestation and the second with FSGS immediately before delivery in week 37. Again, both the cases were treated successfully, with good outcomes for mother and child. One woman developed gestational diabetes and one woman developed gestational hypertension.

From the 29 deliveries, the majority, 24 out of 29 (83%) deliveries were with cesarean section (C-section), in some cases emergent, but in most cases elective, since they comprise the high-risk patient group and obstetricians consider the C-section the safer mode of delivery. Obstetrical complications occurred in three patients: one emergent C-section was performed in one woman in the 37th week due to acute placental hemorrhage, one more woman with twin pregnancy had placenta previa and also underwent emergent C-section, and the third developed an abdominal abscess after delivery with C-section. All the complications were treated successfully with good outcomes for both the women and the neonates.

Renal Parameters

Regarding renal function, most patients in our cohort, 18 out of 22 (82%) patients had excellent renal function at baseline. Median creatinine in the entire cohort was 0.6 (range 0.5–2.0) mg/dl and eGFR (CKD-EPI) was 115 (range 30–120) ml/min/1.73 m^2 at baseline. GFR was calculated with the CKD-EPI equation only at baseline and not during follow-up, since it is unreliable, especially late in the course of pregnancy. Renal function was

TABLE 6 | Maternal outcomes.

	No.	Percentage
Mode of delivery		
Cesarean section	24	82
Vaginal delivery	5	18
Pre-eclamptic syndrome	2	6.7
Nephritis flare	2	7
LN	1	
FSGS	1	
Gestational diabetes	1	3.4
Increase in creatinine (>25% from baseline)	2	7
Gestational hypertension	1	3.4
Obstetric complications	3	10

Percentage per total number of pregnancies (n = 29).

preserved during and immediately after pregnancy as well as after 28 months of follow-up in all the 18 women.

From the remaining four who started pregnancy with creatinine above 1.2 mg/dl, one patient started with 1.3 mg/dl and finished pregnancy with 1.1 mg/dl, while the other three patients deteriorated; more than 25% increase in creatinine was noticed in the first and second patient, with creatinine levels rising from 1.3 to 1.65 mg/dl and from 1.6 to 2.0 mg/dl, respectively, while the third patient had an increase from 2.0 to 2.3 mg/dl. The second and third patient, with the most impaired renal function at baseline, eventually progressed to end-stage renal disease (ESRD) 19 and 48 months after delivery. Trends in creatinine levels are given in **Figures 1**, **2**.

Regarding proteinuria, median baseline proteinuria was low, as expected since most patients were in remission. Median proteinuria was 193 mg/d (range 50–1,590 mg/d) at baseline and did not increase during pregnancy with a median value of 201 mg/d at the end. In 6 out of 29 pregnancies, proteinuria was slightly but not severely elevated, above 1 g/d (range 1.06–1.57 mg/24 h), at pregnancy onset; however, no further increase was noticed. Trends in proteinuria are given in **Figures 3**, **4**.

DISCUSSION

In this single-center study of 29 pregnancies resulting in 30 live births in 22 women with preexisting GD, we could identify a low frequency of adverse maternal and fetal outcomes.

Regarding fetal outcomes, we had a live birth rate approaching 100%, with no neonatal death and only one stillbirth, due to chorioamnionitis, which cannot be attributed to the underlying GD. The rate of low birth weight in our cohort is 33% (7 out of 30 neonates). However, if we exclude the two twin pregnancies, both after ART, resulting in four newborns with very low (below 2 kg) birth weight, the rate is 16.6%, almost twice that of the general population, which for Greece has been reported at 9.3% in 2021 (12). As for twin pregnancies, rates of low birth weight and preterm delivery are 55 and 61% for pregnancies after ART and 50 and 57% in those who have conceived naturally (13). Median duration of pregnancy in our cohort was 37 weeks, with a rate of preterm delivery at 23.4%, again almost twice the rate of the general population of the country, which is at 12% in 2021 (14). From the 30 live newborns, 6 were admitted to the NICU (20%). Again, 4 out of the 6 were the two pairs of twins, who had been admitted due to very low birth weight. One more was admitted due to bradycardia and another due to low birth weight after delivery in the 32nd week because of preeclampsia of the mother. All were discharged without residual impairment.

Our rates were favorable considering prior reported clinical outcomes in women with underlying GD. There are not many studies assessing pregnancy outcomes in women with different subtypes of GD. Regarding former studies, in a review including 6 studies conducted in the late 1980's and comprising a total of 906 pregnancies in 558 women with primary GN, Jungers and Chauveau report a total fetal loss rate at 22% with perinatal (after the 26th week) death of 13% (15). This confirms the findings from smaller studies in the late 1980's to 1990's, which all report high rates of adverse fetal and maternal outcomes. Fetal loss rates are high, with live birth rates about 75% and rates of prematurity, intrauterine growth restriction (IUGR), and low birth weight are in the range of 25–60% (8, 16). Most worryingly, a recent study published in 2017, investigating 48 pregnancies in 43 women with different subtypes of GD, reports also high rates of adverse fetal and maternal outcomes: perinatal death occurred in 12.5%, prematurity rate was 48%, and IUGR at 12.5% (17).

As for maternal outcomes, we had also very good outcomes and few, only minor complications. We found a very low rate of preeclampsia (6.7%) as well as of nephritis flares (7%). Preeclampsia is one of the most common and feared complications of pregnancy in women with kidney disease. Reported preeclampsia rates vary broadly across studies, with the lower rates being about 7–8% and the highest reaching 25%, depending on diagnosis and disease status at conception (18–20). In the most recent study from North Carolina, the rate of preeclampsia was high, at 33% (17). Our pre-eclamptic syndromes were treated with emergent delivery in the 32nd and 36th gestational weeks with successful outcomes for both the mothers and newborns. At this point, it must be noticed that we were in close collaboration with the treating obstetricians and diagnosis of preeclampsia as well as decision about emergent delivery was taken after agreement of both the gynecologist and the nephrologist.

Two nephritis flares occurred during the 29 pregnancies: the first in the 37th gestational week in a patient with FSGS, manifesting with severe nephrotic syndrome, which was successfully handled with urgent delivery and the second in a patient with SLE nephritis, in the 17th gestational week, who developed subnephrotic proteinuria and active urine sediment. In the latter patient as well as in one more patient, with a diagnosis of AASV, who also developed subnephrotic proteinuria and active urine sediment in the 12th gestational week, we performed a renal biopsy. The main clinical indications for renal biopsy during pregnancy are unexpected deterioration of renal function, *de-novo* nephrotic syndrome, and suspicion of new-onset GD. Indications are reasonably strict, since an invasive procedure places both the woman and the embryo at risk. Our

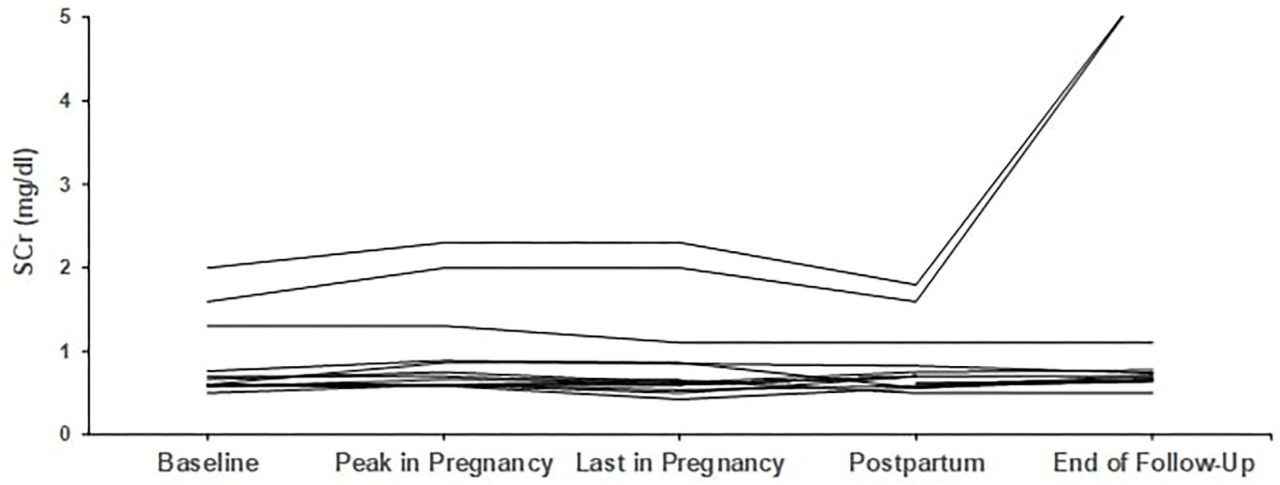

FIGURE 1 | Trends in creatinine in patients with primary glomerular diseases ($n = 13$). Individual trends in serum creatinine levels at five time points: baseline, peak during pregnancy, last pregnancy visit, postpartum, and end follow-up.

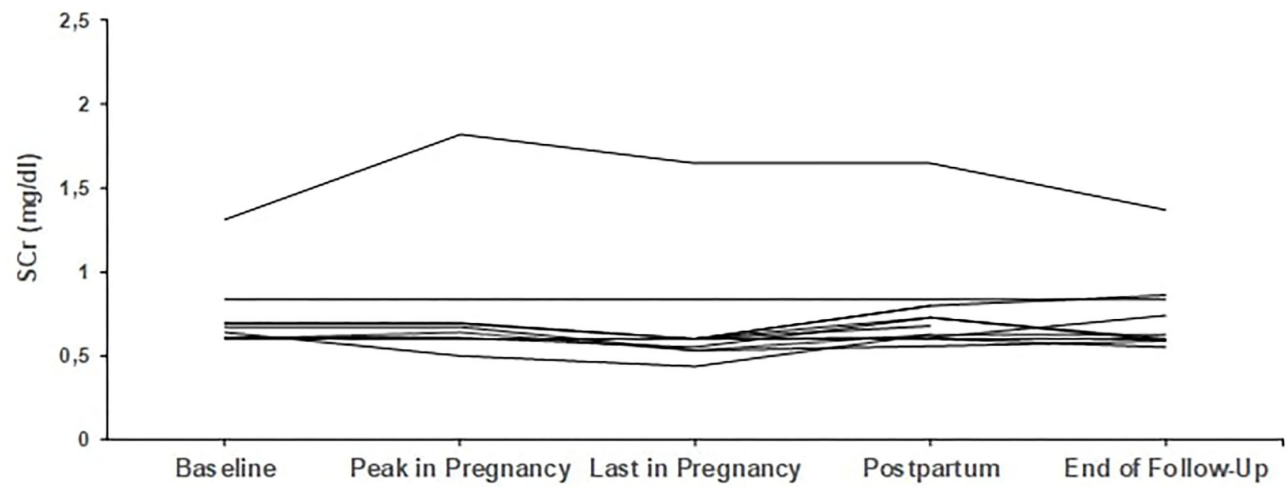

FIGURE 2 | Trends in creatinine in patients with secondary glomerular diseases ($n = 9$). Individual trends in serum creatinine levels at five time points: baseline, peak during pregnancy, last pregnancy visit, postpartum, and end follow-up.

patients had subnephrotic proteinuria and no deterioration of renal function. However, both had reactivated prior inactive urine sediment and suffered from secondary GN, i.e., SLE and AASV, which are both conditions with a high relapse risk. Furthermore, the patient with SLE nephritis had discontinued IS only few days before the onset of pregnancy. In this patient, not surprisingly, kidney biopsy revealed an early nephritis flare with active, though mild-to-moderate class III lesions and she was started IS consisting of azathioprine and steroids. In the second patient with the AASV diagnosis, biopsy revealed only mild mesangial proliferation and a pauci-immune pattern on immunofluorescence; accordingly, no IS was given. Renal biopsy, especially early in the course of pregnancy, when a GD flare is more expected than a pre-eclamptic syndrome, may be extremely helpful for decision guidance in order not only to (re)initiate, but also to withhold otherwise unnecessary treatment. As for safety, both the biopsies were performed in the usual prone position without complications. Regarding complications, the only reliable study reports a low complication rate of 4.5%, comparable to that of the general population, in 111 pregnant women (21). Higher rates are reported late during pregnancy, especially after the 17th gestational week (22).

Renal parameters in means of renal function and proteinuria were excellent at baseline in the majority of patients and remained so until the end of pregnancy and still after 28 months of follow-up. From the three women with creatinine above 1.2 mg/dl at baseline, two women deteriorated and reached ESRD after 19 and 48 months, respectively, and the third women had an

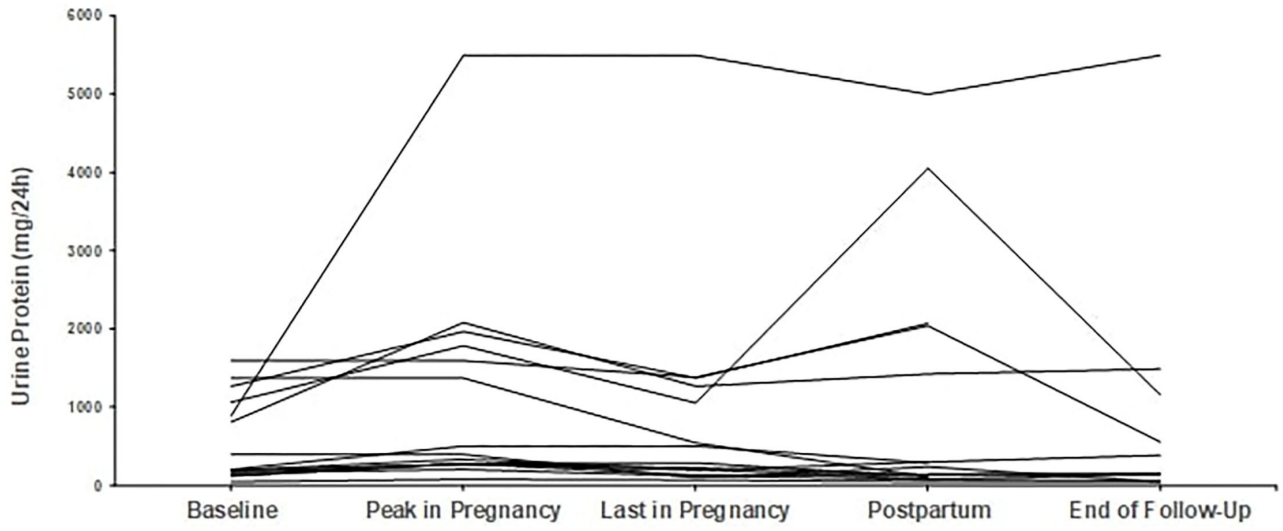

FIGURE 3 | Trends in proteinuria in patients with primary glomerular diseases ($n = 13$). Individual trends in proteinuria at five time points: baseline, peak during pregnancy, last pregnancy visit, postpartum, and end follow-up.

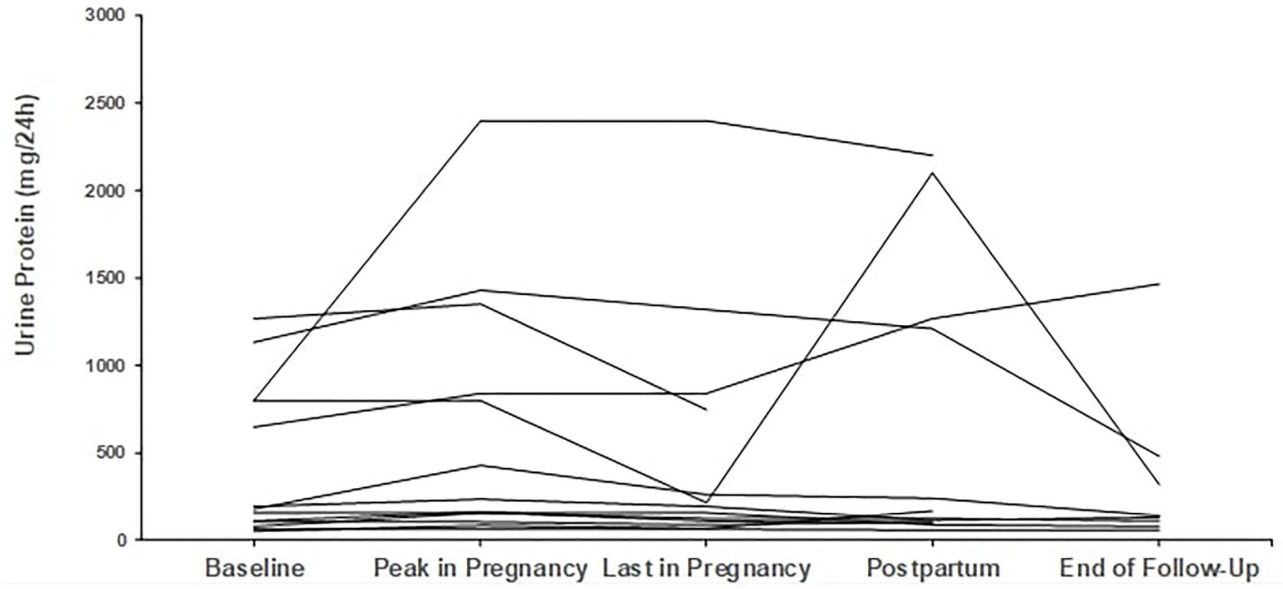

FIGURE 4 | Trends in proteinuria in patients with secondary glomerular diseases ($n = 9$). Individual trends in proteinuria at five time points: baseline, peak during pregnancy, last pregnancy visit, postpartum, and end follow-up.

increase of more than 25% in creatinine, while delivery was before 10 months, so follow-up is still too short to draw conclusions about future outcome. Low proteinuria levels (median of 193 mg/24 h) were also sustained during pregnancy in the 6 women who had started pregnancy with levels above 1 g/24 h and even the effect of hyperfiltration-induced increase in proteinuria was not detected in our cohort. In the abovementioned study from North Carolina, O'Saughnessy and co-authors found a more than 50% increase in creatinine in 27% and doubling of urinary protein in 39% of pregnancies, respectively (17).

Very few studies have evaluated the impact of different GD subtypes on pregnancy outcomes with varying patterns (17, 23). In our cohort, numbers are too small to make such comparisons. However, to our point of view, in secondary GD, but especially in primary GD, main factors that determine successful pregnancy outcomes, more than diagnosis itself, are remission of either nephrotic or nephritic features and preserved renal function at onset.

There are several explanations for the good pregnancy outcomes in our cohort. It is not that our patients had mild

disease. All had long-lasting nephritis with median duration from GN diagnosis until pregnancy of 8.5 years. From the 22 women, 7 women had SLE nephritis and 2 women had AASV, both the conditions associated with a long history, relapses, high immunosuppressive burden, and adverse pregnancy outcomes. Moreover, 82% of patients in this study had received IS for long (median of 58 months), 8 out of them with CYC.

The most critical point is that all the patients were in remission at conception. Quiescent disease is one of the most important features for successful pregnancy outcomes for mothers as for neonates. This is well-documented so far, primarily in SLE, but also in other secondary and primary GD (24–26).

Herein, we must underline the importance of preconception counseling. As already mentioned before, all the patients had long-term follow-up in our institution for preexisting GD. At the preconception visit, the final decision in means of proceeding or not with pregnancy was taken. Many patients had expressed the wish to conceive at different time points during their disease course and had postponed pregnancy if advised so, until it was considered safe. In accordance with this, it is the time interval between GD remission as well as the time interval between IS discontinuation and pregnancy onset; moreover, age of our patients at conception is older compared to that in most other series.

Another important issue for successful pregnancy outcomes in women with underlying GD is preserved renal function. The majority (82%) in our cohort had excellent baseline renal function, which was preserved not only during pregnancy, but also after 28 months of follow-up. Impaired renal function at pregnancy onset has been associated with higher rates of adverse pregnancy outcomes and accelerated maternal CKD. Conversely, in patients with preserved renal function, pregnancy does not negatively influence renal parameters and does not affect long-term renal function (8, 17). This has been shown in a multicenter Italian cohort of 223 pregnant women with primary diagnosis of IgAN, baseline creatinine below 1.2 mg/dl, and follow-up of 10 years (8). The reported outcomes in this cohort are very similar to ours, further supporting the evidence that disease remission and good renal function are major determinants of outcome.

Last but not least, patients in this study had a very close follow-up and there was continuous cooperation between treating physicians of a multidisciplinary team throughout pregnancy.

This study has several limitations: it is a retrospective study from a single institution, with a small number of patients and heterogeneity of the underlying GD. On the other hand, it is a very homogeneous cohort with long-term follow-up before and after pregnancy and close monitoring according to a predefined protocol for laboratory and clinical evaluation at scheduled visits during pregnancy.

CONCLUSION

In women with underlying GDs, pregnancy is safe and should be encouraged, provided they have quiescent disease and preserved renal function.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Data Protection Manager (Laiko General Hospital of Athens, protocol code 118/31-3-2021). The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

SM designed the study and drafted the article. ST, EK, and KV collected and analyzed the data. CS validated the data. SL and JB supervised the process and reviewed the final version of the article. All the authors provided contributions and approved the version of the article to be published.

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frontiers in Medicine

BRIEF RESEARCH REPORT
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Plasma Homocysteine as a Potential Marker of Early Renal Function Decline in IgA Nephropathy

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Hyperhomocysteinemia (HHcy) is very common among patients with chronic kidney disease (CKD), and related to the risk of cardiovascular events and mortality in these patients. However, the prevalence of HHcy in primary causes of CKD and its role in kidney disease progression are not well-understood. In this study, we investigated the prevalence of HHcy in different CKD stages in 221 patients with IgA nephropathy (IgAN) and 194 patients with other primary glomerular diseases. We also evaluated the association of homocysteine (Hcy) [after adjusted for estimated glomerular filtration rate (eGFR)] with CKD progression event, defined as ESKD or 50% decline in eGFR, in a cohort of 365 patients with IgAN. The prevalence of HHcy was 67.9% (150/221), 53.5% (76/142), 51.5% (17/33), and 42.1% (8/19) in patients with IgAN, membranous nephropathy, minimal change disease, focal segmental glomerulosclerosis, respectively. The Hcy/eGFR ratio was significantly associated with pathologic features of IgAN, including the proportion of global glomerulosclerosis ($r = 0.38$, $p < 0.001$), the proportion of ischemia originated glomerular sclerosis ($r = 0.32$, $p < 0.001$), and the severity of tubular atrophy/interstitial fibrosis ($r = 0.57$, $p < 0.001$). Importantly, Hcy/eGFR ratio was an independent risk factor for CKD progression event (hazard ratio, 1.38; 95% confidence interval, 1.13–1.68; $p = 0.002$). The risk of CKD progression events continuously increased with the Hcy/eGFR ratio, but reached a plateau when Hcy/eGFR ratio was >1.79. Our findings suggest that elevated Hcy/eGFR ratio may be an early marker of poor renal outcome in IgAN.

Keywords: IgA nephropathy, homocysteine, chronic kidney disease, primary glomerular diseases, kidney disease progression

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INTRODUCTION

Primary glomerular diseases (PGD)—glomerular diseases that are not caused by a systemic disease—account for about 20% of chronic kidney disease (CKD) in most countries (1). A great number of PGD are "silent" at onset of the disease, and are diagnosed on urinary tests during routine medical examination (2). Unlike other causes of CKD such as diabetes and hypertension, they frequently affect young people with associated morbidity and high cost. IgA nephropathy (IgAN) is the most common PGD worldwide and the most common cause of ESKD among Asian populations (3). IgAN is currently monitored by the presence of proteinuria and/or changes

in serum creatinine indicating decline in glomerular filtration rate (GFR). However, the lack of reproducibility in albuminuria measurements and high variability in GFR at mild to moderate stages of disease have raised concerns about their ability to accurately represent CKD progression.

Homocysteine (Hcy) is a sulfur-containing intermediary amino acid produced following the metabolic conversion of methionine to cysteine. Hyperhomocysteinemia (HHcy) is a condition characterized by the increase in plasma levels of Hcy—total plasma levels of Hcy >15 μmol/L. Hcy can reduce the utilization activity of nitric oxide, increase oxidative stress, induce endothelial dysfunction, and promote the proliferation of vascular smooth cells (4, 5). Raised plasma total Hcy is an independent risk factor for cardiovascular disease and atherothrombosis (6). Patients with CKD have markedly raised Hcy, and the risk of cardiovascular events and mortality is related to the concentration of Hcy (7). Folic acid therapy could significantly delay the progression from normal to moderate CKD in patients with hypertension (8). However, the role of Hcy levels in disease progression in patients with PGD remains unclear.

In this study, we enrolled 221 cases with IgAN and 194 cases with other PGD to investigate the prevalence of HHcy in different CKD stages in these patients. We also evaluated the association of Hcy/eGFR ratio with disease severity and CKD progression in a cohort of 365 patients with IgAN.

MATERIALS AND METHODS

Study Participants

The cross-sectional study was based on data obtained from medical records from the renal division of Peking University First Hospital. We included participants with PGD who underwent comprehensive health examinations, including renal biopsy and plasma concentrations of total Hcy from January 2016 to December 2020. We excluded individuals with gout, pyuria, or nephritis secondary to systemic disease or cancer, as well as other factors that influence the plasma Hcy levels. Individuals with gastrointestinal diseases, alcoholic addiction, vegetarian lifestyle, and regular consumption of folic acid and vitamin supplements were also excluded. Finally, 221 cases with IgAN were recruited in the observational study. The diagnosis was based on renal biopsy by light microscopy, immunofluorescence, and electron microscopy. At the same time interval, 142 patients with membranous nephropathy (MN), 33 patients with minimal change disease (MCD), and 19 patients with focal segmental glomerulosclerosis (FSGS) were enrolled served as disease controls.

In addition, to explore the role of Hcy in disease progression, a total of 365 patients with biopsy-proven IgAN diagnosed between 2009 and 2020 were selected from the prospective IgAN database at Peking University First Hospital. Patients in the database were followed up regularly every 3–6 months.

This study was approved by the Ethics Committee of Peking University First Hospital and informed written consent was obtained from all patients.

Data Collection

Clinical manifestations at the time of renal biopsy, including age, sex, homocysteine, serum creatinine, 24-h urine protein excretion, uric acid, and other biochemical characteristics were collected from the medical records. The definition of HHcy was referred to as the presence of homocysteine concentrations >15 μmol/L. The eGFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation (9). The Oxford classification of IgAN was used for the evaluation of pathologic lesions for those with more than eight glomeruli in biopsy specimens (10). The CKD progression event was defined by a 50% decline in eGFR or ESKD that was confirmed by a second evaluation obtained at least 4 weeks later.

Statistical Analyses

Continuous variables were presented as mean ± standard deviation for normally distributed variables or median with interquartile range for non-normally distributed variables. Categorical variables were summarized as frequency with percentage. For continuous variables, the significance of differences between groups was determined using independent sample *t*-test. A Mann-Whitney *U*-test was used to compare the differences between two independent samples when the sample distributions are not normally distributed. Differences among categorical variables were analyzed using the chi-squared test.

Cox proportional hazards models were adopted to evaluate the relationship between Hcy/eGFR ratio and risk of end point. Sex, age, eGFR, proteinuria, mean arterial pressure, and use of steroids and/or other immunosuppressive agents were adjusted in multivariable-adjusted Cox proportional hazards models. Receivers operating characteristic (ROC) curves were constructed at the most discriminating cut-off point value to predict the disease progression. Kaplan-Meier analysis was used to derive cumulative kidney survival curves. The division between the groups of participants was on the basis of the cut-off value in the ROC curves, and difference between curves was analyzed using a log-rank test. The association between Hcy/eGFR ratio and end point was also evaluated on a continuous scale with restricted cubic spline curves based on Cox proportional hazards models. Knots for the cubic splines were placed with default parameters.

To investigate correlations between Hcy/eGFR ratio and clinicopathological parameters in IgAN patients, Pearson's correlation coefficients were used for continuous variables and Spearman's correlation coefficients for binary and ordinal variables. Partial correlation analysis was used to quantify the correlation between two variables after removing the effects of other variables. All *p*-values were 2 tailed, and values <0.05 were considered statistically significant. Analyses were performed using SPSS software v20.

RESULTS

Association of Hyperhomocysteinemia With Reduced Renal Function

The characteristics of the patients with PGD at the time of kidney biopsy are described in **Table 1**. In the cross-sectional study, we

TABLE 1 | Baseline characteristics of patients with different primary glomerular diseases.

Characteristics	IgAN ($n = 221$)	MN ($n = 142$)	MCD ($n = 33$)	FSGS ($n = 19$)
Age (years)	37.7 ± 11.6	52.1 ± 16.1	46.0 ± 19.1	38.5 ± 15.4
Sex (men, %)	117 (52.9)	92 (64.8)	18 (54.5)	15 (78.9)
Homocysteine (μmol/L)	19.1 (14.1–27.2)	16.0 (10.2–24.8)	17.1 (9.6–27.1)	13.7 (11.6–27.4)
Serum creatinine (μmol/L)	111.0 (81.0–166.2)	91.8 (65.3–128.3)	90.9 (64.9–164.1)	127.5 (82.9–212.6)
eGFR (mL/min per 1.73 m^2)	68.1 (40.2–91.0)	78.0 (47.2–100.8)	76.1 (36.8–109.6)	61.0 (29.0–88.1)
Proteinuria (g/24 h)	1.4 (0.6–3.0)	3.3 (1.3–7.0)	3.0 (1.0–7.1)	1.8 (0.5–7.0)
Serum uric acid (μmol/L)	403.7 ± 106.6	390.4 ± 116.0	411.9 ± 123.7	412.1 ± 96.9
Blood urea nitrogen (mmol/L)	8.2 ± 5.1	8.5 ± 5.2	8.5 ± 6.5	11.4 ± 9.2
Folic acid (nmol/L)	29.6 (18.1–53.9)	17.4 (13.3–52.2)	21.4 (12.8–53.5)	33.1 (17.5–53.5)
Vitamin B12 (pg/mL)	245.5 (166.3–337.0)	255.0 (186.0–355.0)	220.0 (174.5–442.8)	292.0 (148.5–639.5)
Treated with steroids or other immunosuppressives (*n*, %)	46 (20.8)	78 (54.9)	32 (97.0)	19 (100.0)

Data are presented as n (%), mean ± SD, or median (25th−75th percentile). IgAN, IgA nephropathy; MN, membranous nephropathy; MCD, minimal change disease; FSGS, focal segmental glomerulosclerosis; eGFR, estimated glomerular filtration rate.

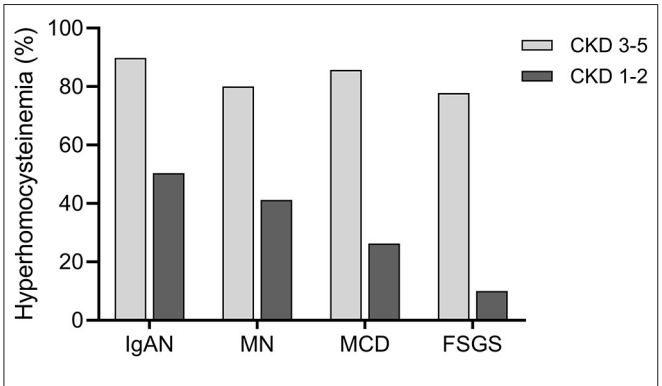

FIGURE 1 | Prevalence of hyperhomocysteinemia (HHcy) in different CKD stages in primary glomerular diseases. In patients with CKD stages 3–5, the prevalence of HHcy was 89.8% (88/98), 80.0% (36/45), 85.7% (12/14), and 77.8% (7/9) in IgAN, MN, MCD, and FSGS, respectively. In patients with CKD stages 1–2, the prevalence of HHcy was 50.4% (62/123), 41.2% (40/97), 26.3% (5/19), and 10.0% (1/10) in IgAN, MN, MCD, and FSGS, respectively.

randomly enrolled 221 cases with biopsy proven IgAN. The mean age of IgAN was 37.7 ± 11.6 years, and men accounted for 52.9% of the cohort. At the time of diagnosis, the average eGFR was 66.6 ± 32.5 mL/min per 1.73 m^2 and the median proteinuria level was 1.4 g/24 h (interquartile range, 0.6–3.0 g/24 h).

The mean homocysteine level in IgAN was 23.2 ± 15.3 μmol/L (range, 7.1–100.0 μmol/L). HHcy was detected in 150 (67.9%) of the 221 patients in IgAN group. Whereas, the prevalence of HHcy was 53.5% (76/142), 51.5% (17/33), and 42.1% (8/19) in MN, MCD and FSGS groups, respectively. As shown in **Supplementary Table 1**, individuals with HHcy presented with more severe disease, including higher serum creatinine ($p < 0.001$) and uric acid levels ($p < 0.001$), and lower eGFRs ($p < 0.001$) than those without HHcy, which was not specific to any kind of PGD.

We also explored the prevalence of HHcy in different CKD stages (CKD stages 1–2 vs. 3–5) in IgAN and disease controls (**Figure 1**). In CKD stages 1–2, the prevalence of HHcy was 50.4, 41.2, 26.3, and 10.0% in IgAN, MN, MCD, and FSGS, respectively ($p = 0.02$). In CKD stages 3–5, the prevalence of HHcy was 89.8, 80.0, 85.7, and 77.8% in IgAN, MN, MCD, and FSGS, respectively ($p = 0.38$). Specifically, the prevalence of HHcy in CKD stages 1–2 in IgAN was 36.2% (21/58) and 63.1% (41/65), respectively. Thus, HHcy is very common in IgAN cases of normal or mildly reduced renal function (about 1/3 in CKD 1 and 2/3 in CKD 2), which may be of clinical importance. In line with expectations, the prevalence increased with decreasing renal function, which was 84.4% (54/64), 100.0% (28/28), and 100.0% (6/6) in CKD stages 3, 4, and 5 in IgAN, respectively (**Supplementary Table 2**).

Association of Hyperhomocysteinemia With Clinicopathologic Characteristics in IgAN

Then, we explored the correlations between Hcy (after adjusted for eGFR) and clinicopathological parameters in patients with IgAN (**Figure 2**, **Supplementary Table 3**). The Hcy/eGFR ratio showed a negative correlation with eGFR ($r = -0.65$, $p < 0.001$), and remained significant after controlling for cardiovascular risk factors, including age, sex, hypertension, and total cholesterol levels ($r = -0.63$, $p < 0.001$). The Hcy/eGFR ratio was also positively correlated with serum creatinine ($r = 0.76$, $p < 0.001$), serum uric acid ($r = 0.35$, $p < 0.001$), proteinuria ($r = 0.10$, $p = 0.03$), systolic blood pressure ($r = 0.13$, $p = 0.01$), and diastolic blood pressure ($r = 0.11$, $p = 0.03$). In addition, the Hcy/eGFR ratio was significantly associated with pathologic features of IgAN, including the mesangial hypercellularity ($r = 0.20$, $p < 0.001$), the segmental glomerulosclerosis ($r = 0.14$, $p = 0.01$), the proportion of global glomerulosclerosis ($r = 0.38$, $p < 0.001$), the proportion of ischemia originated glomerular sclerosis ($r = 0.32$, $p < 0.001$), the severity of tubular atrophy/interstitial fibrosis ($r = 0.57$, $p < 0.001$), and the presence of crescent ($r = 0.15$, $p = 0.01$). These results suggest that Hcy might contribute to the progression of IgAN.

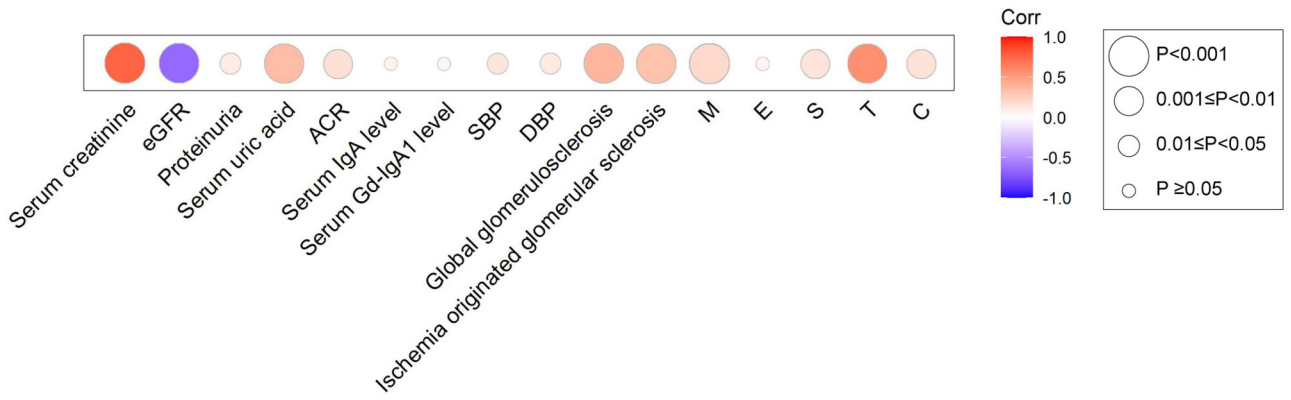

FIGURE 2 | Heatmap for the association between Hcy/eGFR ratio and clinical and pathologic manifestations in IgAN. Circles were colored by the correlation coefficients, and the size of the circle represents correlation significance level.

TABLE 2 | Hcy/eGFR ratio as a risk factor for the composite kidney disease progression event in IgAN.

	Hazard ratio for composite outcome (95% confidence interval); *p*			
	Unadjusted	**Model 1[b]**	**Model 2[c]**	**Model 3[d]**
CKD progression event[a], per 1 s.d. Hcy/eGFR	1.41 (1.27–1.58) 8.11×10^{-10}	1.46 (1.30–1.65) 5.14×10^{-10}	1.23 (1.06–1.42) 0.007	1.38 (1.13–1.68) 0.002

[a] *CKD progression event was defined as a 50% decline in eGFR or ESKD.*
[b] *Model 1 was adjusted for sex and age. Sex was analyzed as dichotomous data.*
[c] *Model 2 was adjusted for covariates in model 1 plus eGFR, proteinuria, and mean arterial pressure.*
[d] *Model 3 was adjusted for covariates in model 2 plus steroids or other immunosuppressive agents. Use of treatment with steroids and/or other immunosuppressive agents was analyzed as dichotomous data.*

Association of Hcy/EGFR Ratio With Kidney Disease Progression in IgAN

The clinical and pathologic characteristics of IgAN cohort are summarized in **Supplementary Table 4**. At baseline there were 199 (54.5%) men and mean age was 39.1 ± 12.7 years. eGFR was 70.6 ± 33.0 mL/min per 1.73 m^2 and the median proteinuria level was 1.1 g/24 h (interquartile range, 0.6–2.4 g/24 h). The mean homocysteine level in IgAN was 23.7 ± 19.4 μmol/L (range, 4.6–140.9 μmol/L). Overall, 238 participants (65.2%) received steroids or other immunosuppressive agents. After a median follow-up of 34 months (IQR, 22–49 months), 32 (8.8%) participants reached the composite kidney disease progression event, including 27 kidney failure events. Compared with other patients, these patients showed a higher prevalence of HHcy (90.6 vs. 64.3%; $p = 0.003$) and more severe histological lesions, including higher prevalence of M1 (65.6 vs. 43.2%; $p = 0.02$), S1 (87.5 vs. 58.9%; $p = 0.001$), and T1/T2 (96.9 vs. 38.7%; $p < 0.001$) according to the Oxford classification (**Supplementary Table 5**).

As shown in **Table 2**, in cause-specific hazards models, Hcy/eGFR ratio was an independent risk factor for the composite kidney disease progression event in model 3 [hazard ratio (HR), 1.38; 95% confidence interval (95% CI), 1.13–1.68; $p = 0.002$] after adjustment for sex, age, baseline eGFR, proteinuria, mean arterial pressure, and use of steroids or other immunosuppressive agents. The ROC curves showed discriminatory power of Hcy/eGFR ratio on disease progression (**Supplementary Figure 1**). The area under the ROC curves (AUC) was 0.79 with the cut-off point of 0.46. Then, Kaplan-Meier curves demonstrated that patients with Hcy/eGFR ratio >0.46 had a higher incidence of the composite kidney disease progression event (log-rank test, $p < 0.001$) (**Figure 3A**).

The association between Hcy/eGFR ratio and end point was also evaluated on a continuous scale with restricted cubic spline curves based on Cox proportional hazards models (**Figure 3B**). The risk of CKD progression events continuously increased with the Hcy/eGFR ratio, but reached a plateau when Hcy/eGFR ratio was >1.87. The non-linear association was also found in the multivariable-adjusted model, with a plateau when Hcy/eGFR ratio was >1.79.

DISCUSSION

In this study, we investigated the prevalence of HHcy in 221 patients with IgAN and 194 patients with other primary glomerular diseases, and found that patients with HHcy tended to present with more severe disease than those without HHcy. Among the four kinds of PGD, IgAN had the highest prevalence of HHcy, especially in patients with CKD stages 1–2. Notably, our study demonstrated that Hcy/eGFR ratio was associated with the chronic changes in kidney biopsy specimens in IgAN. On the basis of our follow-up data, we found that elevated Hcy/eGFR

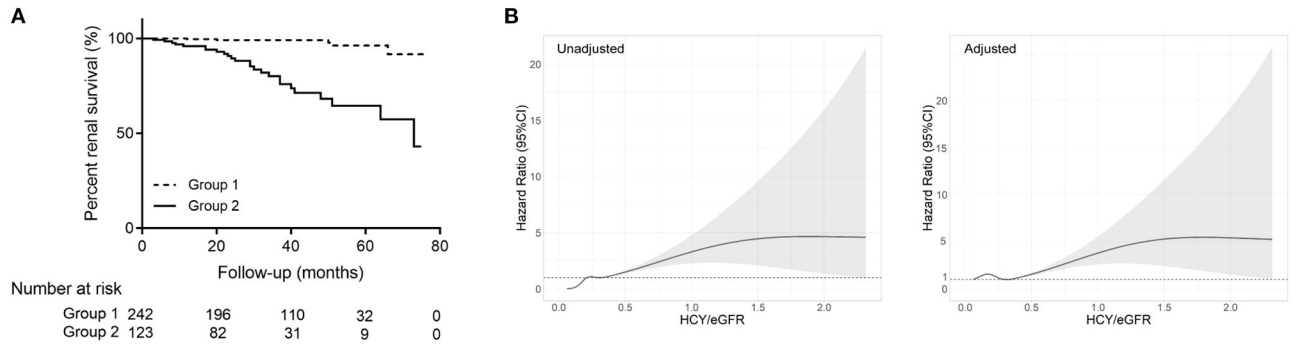

FIGURE 3 | Association of Hcy/eGFR ratio with kidney disease progression in IgAN. **(A)** Kaplan-Meier kidney survival curves of participants with IgAN according to Hcy/eGFR ratio. The time zero was kidney biopsy. The division between the groups of participants was on the basis of the cut-off value of Hcy/eGFR ratio (Group 1: low Hcy/eGFR ratio; Group 2: high Hcy/eGFR ratio). **(B)** Unadjusted and multivariable-adjusted hazard ratios for CKD progression events according to Hcy/eGFR ratio on a continuous scale. The solid lines represent the estimated hazard ratios, with the shaded areas showing 95% confidence intervals derived from restricted cubic spline regressions with five knots. Reference lines for no association are indicated by the dashed lines at a hazard ratio of 1.0. The model was adjusted for age, sex, eGFR, proteinuria, mean arterial pressure, and use of steroids and/or other immunosuppressive agents.

ratio was independently associated with increased risk of kidney disease progression in IgAN.

The prevalence of HHcy in patients with CKD has been reported (11). Nevertheless, the different primary causes of CKD have not been considered in these analyses. The present study found that HHcy was most pronounced in patients with IgAN, of which 67.9% with Hcy > 15 μmol/L. Furthermore, the higher Hcy levels in IgAN were not due to gender distributional differences: men accounted for 52.9% of IgAN, the lowest among the four primary glomerular diseases. The association between Hcy and IgAN was independent of eGFR, as there was no significant difference in eGFR between IgAN and disease controls. Consistent with previous data, we found that patients with advanced CKD had an increased prevalence of HHcy. In addition, the prevalence differences of HHcy among primary glomerular diseases mainly existed in the early stages of CKD.

Moreover, we found that Hcy/eGFR ratio was significantly associated with eGFR, proteinuria, and multiple chronic pathologic features of IgAN, which are important markers for the development and progression of CKD. Thus, the Hcy/eGFR ratio may be a potential marker for predicting the prognosis of IgAN. Consistently, participants reached the CKD progression event presented more severe histological lesions, including mesangial hypercellularity, segmental glomerulosclerosis, and tubular atrophy/interstitial fibrosis. Chronic changes in kidney biopsy specimens have a major bearing on predicting prognosis of CKD and guiding treatment (12). Endocapillary hypercellularity is one of the pathologic features of IgAN that was found to be associated with disease severity (13). Repeat biopsies indicated that the progression of glomerular sclerosis is dependent on the degree of glomerular endothelial proliferation at the first biopsy (14). Chronic elevated plasma Hcy level alters functions of the vascular endothelial cells, which compromises the integrity of the vessel wall and in turn the vascular tone, leading to vascular inflammation (15). Therefore, early intervention of HHcy in IgAN is essential to prevent progression to CKD and ESKD, especially for those with glomerular endothelial injury.

A previous study has reported the association between Hcy levels and renal function decline in hypertensive adults (16). To our knowledge, we first estimated the role of Hcy in kidney disease progression in patients with IgAN. The Hcy/eGFR ratio was independently associated with the composite kidney disease progression event in IgAN during the follow-up. Similar results were obtained based on restricted cubic spline curves, which allowed for flexibility examining in the association between continuous Hcy/eGFR ratio and the risk of CKD progression events. However, our study has several limitations. First, this was a single-center study with relatively small sample size, and the findings need to be confirmed in other populations. Second, the follow-up was relatively short (with a median of ∼3 years), during which few end points were observed.

In conclusion, HHcy was more prevalent in IgAN patients than in patients with other primary glomerular diseases, especially in the early stages of CKD. Hcy/eGFR ratio was significantly associated with markers of poor renal outcome in IgAN. Importantly, we use a cohort study demonstrating that elevated Hcy/eGFR ratio was independently associated with increased risk of disease progression in IgAN. Further high-quality trials of early intervention of HHcy in IgAN to prevent progression to CKD and ESKD are warranted.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the Ethics Committee of Peking University First Hospital. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

X-JZ and HZ designed the study. Y-NW carried out experiments and data analysis. HX and Z-RS contributed to sample collection and performed clinical characterization. All authors contributed to the manuscript and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmed.2022.812552/full#supplementary-material

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ORIGINAL RESEARCH
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SUMO1 Promotes Mesangial Cell Proliferation Through Inhibiting Autophagy in a Cell Model of IgA Nephropathy

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IgA nephropathy (IgAN) is a common form of primary glomerulonephritis and its main pathological changes are mesangial cell proliferation and matrix expansion. Autophagy inhibition may result in its mesangial cell proliferation and renal lesions. SUMOylation is a eukaryotic-reversible post-translational modification where SUMO is covalently attached to target proteins to regulate their properties. It is largely unclear whether SUMOylation contributes to the pathogenesis of IgAN. This study was designed to investigate the change of protein SUMO1 in mesangial cells of IgAN and its association with autophagy. We found the expression of SUMO1 was upregulated in IgAN, IgA mouse model, and aIgA1-stimulated mesangial cells. In aIgA1-stimulated mesangial cell model, we tested LC3II/I and p62, the autophagy-related proteins suggested the inhibition of autophagy. Inhibited SUMOylation with ginkgolic acid (GA) or silencing SUMO1 could downregulate SUMO1 and SUMO1-p53, promote autophagy, and lessen cell proliferation. In summary, in the mesangial cells stimulated with aIgA1, SUMO1 may contribute to its cell proliferation through inhibited autophagy, and SUMO1-p53 may play a role in this process.

Keywords: SUMO1, autophagy, IgA nephropathy, mesangial cell, proliferation

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INTRODUCTION

IgA nephropathy (IgAN) is the most common form of primary glomerulonephritis and an important cause of chronic kidney disease and end-stage kidney failure worldwide. A central finding in patients with IgAN is the presence of circulating and glomerular immune complexes comprised of galactose-deficient IgA1. It is characterized by mesangial cell proliferation and mesangial matrix expansion (1). In addition to its well-known multi-hit pathogenesis (2, 3), many factors such as complement activation and autophagy are involved in its occurrence and development (4, 5). Although the pathogenesis of IgAN has been explored at various cellular and molecular levels, no information on protein SUMOylation has been reported in IgAN.

Protein post-translational modifications (PTMs) play essential role in various biological functions. SUMOylation is one of PTMs. SUMOylation modifications are reversible and dynamic processes, in which the modified proteins can be deSUMOylated by SUMO-specific proteases. SUMO includes a family of peptide of 11 kDa, which consists of four informs: SUMO1, SUMO2, SUMO3, and SUMO4. The forms of SUMO2 and SUMO3 are 95% identical in sequence, so they

are often grouped together as SUMO2/3 (6). SUMOylation plays crucial roles in chromosomal organization and function, genome stability, proteasomal degradation of proteins and DNA damage repair, and quality control of newly synthesized proteins (7). There is abundant evidence to show that the aberrance of SUMOylation regulation is highly associated with various proliferative diseases that include cancer. But there is no information on protein SUMOylation, which has been reported in IgAN.

Autophagy is a self-degradative process that represents an important physiological catabolic mechanism of the eukaryotic cell. The process of autophagy includes phagophore, autophagosome formation, fusion and degradation, and recycling (8). In the recent years, studies have shown that autophagy plays a crucial role in various cell types, which include neurons, muscles, and cancer cells (9). The molecular control of autophagic activation is dominated by tumor suppressor and oncogene proteins that functionally represent protein kinases (10). The key tumor suppressor protein p53 has numerous tasks, which include regulating cell cycle and autophagy (11). p53 controls the process of autophagy by its own activation or deactivation. By different intracellular positioning, p53 plays diverse roles in autophagy regulation: p53 facilitates autophagy when positioned in the cell nuclei because it can transactivate the two subunits of AMPK as well as TSC2 (12, 13); when positioned in the cytoplasm, p53 inhibits autophagy in three ways: activating autophagy inhibitor mTOR, inhibiting the effect of AMPK, and exerting a direct effect (14, 15). p53 is the target of SUMOylation, and SUMO1 could mediate its transactivation and apoptosis (16). In IgAN, p53 is upregulated in intrinsic renal cells (17). Although the relationships between human disease and autophagy are complicated, there is growing evidence that autophagy is involved in kidney aging and the pathogenesis of kidney disease. In IgAN, the previous study suggested that autophagy inhibition may result in mesangial cell proliferation and renal lesions (5). There are also some studies that showed that the SUMOylation of some proteins is required for autophagosome creation in autophagy and its effects on the regulation of autophagy are complicated (18, 19).

Based on the previous findings, we hypothesized that SUMO1 can promote mesangial cell proliferation through p53's effection of inhibiting autophagy.

MATERIALS AND METHODS

Ethics Statement

The study was approved by the clinical experiment ethical committee of The Second Xiangya Hospital of Central South University, and informed consent was obtained from all study subjects.

Specimen Collection

Specimens were collected from the patients who were taken renal biopsy in our hospital. A number of 10 patients with IgAN and 10 adjacent patients with minor glomerular abnormality were diagnosed by clinical manifestation and renal biopsy.

Animals and Experimental IgAN Model

A total of 22 Balb/c rats were purchased from the Hunan SJA Laboratory Animal Co. Ltd. (China) and were bred under controlled environmental conditions. All animal experiments were performed in accordance with the protocols of the Institutional Animal Care and Use Committee at Central South University.

After 1 week of adaptation with general meals and diet balance, the rats were randomly distributed into 2 groups: model group and blank group. The method inducted a successful rat model of IgAN based on our previous studies (5). In brief, rats were induced with continuous oral immunization containing bovine serum albumin (BSA) (Sigma) 800 mg/kg, HCl 8.4 mmol/l in tap water for 18 weeks, followed by subcutaneous injection of castor oil and carbon tetrachloride (CCl_4) 0.1 ml (5:1) once per week and intraperitoneal injection 0.06 ml once every 2 weeks. In addition, lipopolysaccharide (LPS, 50 μg) was injected *via* tail vein at the 6th and 8th week. For the control group, saline replaced the solvent. The IgAN model was established at the end of the 18th week.

Immunohistochemical Staining

All the renal staining was performed on 4-μm paraffin sections as we described previously (5). Immunohistochemical stains were performed on formalin-fixed, paraffin-embedded 4-μm sections. Sections were rehydrated and antigens retrieved using heated citrate; primary antibodies were used against the following proteins: SUMO1 (rabbit anti-human, rat, 1:1,000, Cell Signal Technology) or integrin-α8 antibody (mouse anti-human, 1:100, R&D Systems). We used PBS instead of the primary antibodies for negative controls. Then, staining was visualized using horseradish peroxidase (HRP)-coupled secondary antibodies (goat anti-rabbit, 1:500; Abcam). For immunofluorescence, secondary antibodies were coupled to fluorochromes, and nuclei were stained with DAPI (Sigma). The images were acquired using a fluorescence microscope (Nikon Tokyo, Japan) or a confocal laser scanning microscope (Nikon Tokyo, Japan). All immunohistochemical and immunofluorescence analyses were repeated at least 3 times and representative images were presented.

Cell Culture and Treatment

Human mesangial cells (HMCs) were obtained from the Cell-Bio company and cultured in DMEM supplemented with 10% fetal bovine serum (FBS, Gibco, USA), 100 IU/ml of penicillin, and 100 mg/ml of streptomycin. All cells were maintained at 37°C in a humidified atmosphere with 5% CO_2. Human mIgA1 was purchased from Abcam company. We incubated the purified mIgA1 at 63°C for 150 min to obtain aggregated IgA1(aIgA1) as described previously (20, 21). The transition from mIgA1 to aIgA1 was monitored using a Sephacryl S-200 column, and a single peak was observed after incubated at 63°C. After cell growth was arrested for 24 h without FBS, HMCs were incubated for 24 h with 25 μg/ml aIgA1 and 24 h with 2 μmol/l ginkgolic acid (GA) or with PBS as control.

Small Hairpin RNA (shRNA) Plasmids and Cell Transfection

In view of the established characteristics of siRNA-targeting constructs, we designed a pair of siRNA oligonucleotides for SUMO1:5′-GTGACAACACATCTCAAGAATTCAAGAGATTCTTGAGAT GTGTTGTCATTTTTT-3′. The cells were transfected by Lipofectamine 3000 liposome transfection reagent kit of Invitrogen company. HMCs were transfected using control shRNA or shRNA against SUMO1 constructed by Vigene Biosciences Company, following the protocols provided by the manufacturer.

Real-Time PCR

Total RNA was isolated from cells with TRIzol Reagent (Invitrogen, USA). The first-strand cDNA synthesis was performed using a PrimeScript^{TM} RT reagent kit (Takara, Japan). Real-time PCR was performed to determine relative mRNA levels using a SYBR^{R} Premix Ex Taq^{TM} II (Takara, Japan) on the LightCycler^{R} 96 PCR system (Roche, Switzerland). PCR cycling conditions included an initial step at 95°C for 30 s, followed by 40 cycles of 5 s at 95°C, and 30 s at 60°C. The PCR products were assessed by melting curve analysis, and gene expression levels were calculated using the $\Delta\Delta C_t$ method after normalization to the GAPDH housekeeping gene. All PCR samples were tested in triplicate.

Western Blotting

Cells from different groups were collected, lysed in RIPA Lysis Buffer (Beyotime Biotechnology), and centrifuged at 12,000 g for 10 min at 4°C. The supernatants were collected, and the cellular protein concentrations were determined with a BCA protein assay kit. Protein samples were denatured at 95°C for 5 min, separated by SDS-PAGE, and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore). The blots were incubated with primary antibodies against SUMO1 (1:1,000, rabbit anti-human, Cell Signal Technology), SUMO2/3 (1:1,000, rabbit anti-human, Cell Signal Technology), p53 (1:1,000, mouse anti-human, Cell Signal Technology), cyclin D1 (1:200, rabbit anti-human, Abcam), LC3 (1:1,000, rabbit anti-human, Cell Signal Technology), p62 (1:1,000, rabbit anti-human, Proteintech), or β-actin (1:1,000, mouse anti-human, Abcam) overnight at 4°C. Subsequently, the blots were incubated with an HRP-conjugated secondary antibody (goat anti-rabbit IgG H&L and goat anti-mouse IgG H&L) at room temperature for 1 h, and then, enhanced chemiluminescence was used to visualize the bands.

Immunoprecipitation

Cell pellets were washed three times with cold PBS and lysed in RIPA buffer for 30 min on ice. After centrifugation at 12,000 rpm for 15 min at 4°C, the lysates were collected and precleared by Protein A/G Plus-Agarose (Santa Cruz Biotechnology) and then incubated with an anti-p53 antibody (1:500, mouse anti-human, Cell Signaling Technology) with continual shaking for 1 h. The protein-antibody complexes were collected with 20 μl of protein A/G plus agarose at 4°C with continual shaking overnight. The next day, the immunoprecipitates were washed three times with lysis buffer and analyzed by SDS-PAGE and immunoblotting.

Flow Cytometer

Cells in different treatment groups were isolated, fixed in 70% cold ethanol, and stored overnight at −20°C. After washing with PBS, propidium iodide (PI) staining solution (50 μg/ml PI and 100 μg/ml RNase A) was added to the cells and incubated for 30 min in the dark at 37°C. Then, cells were analyzed using flow cytometry (Becton Dickinson Biosciences, USA). FlowJo software was used to analyze the results. Three independent experiments were conducted.

Statistical Analysis

All statistics were analyzed by SPSS 19.0 statistical software and GraphPad Prism 6.0. Continuous variables are expressed as mean ± standard deviation. The cellular experiments were repeated 5–10 times, and the animal experiments were replicated 5–10 times. Comparison between two groups was detected by *t*-test, whereas comparison among multiple groups was detected by one-Way ANOVA and Kruskal–Wallis for non-parametric test. Difference was considered as statistically significant if $p < 0.05$.

RESULT

Comparison of SUMO1 Expression Between IgAN Kidney Tissues and Adjacent Tissues

Immunofluorescence staining against integrin- α8, a mesangial marker, was used to examine the intraglomerular localization of SUMO1 (**Figure 1A**). SUMO1-positive areas in the human glomeruli were mostly overlaid with integrin- α8 staining, which suggests the mesangial cellular localization of SUMO1.

Immunohistochemistry staining for SUMO1 indicated that SUMO1 was expressed in human mesangial cells. The expression of SUMO1 was upregulated in IgAN patients' group than the minor glomerular abnormality control group (**Figure 1B**).

In IgAN mouse model, immunofluorescence demonstrated mesangial IgA deposition. The PAS and HE staining in **Figure 1** provide a characteristic overview of the pathologic changes in glomerulus. Compared with control mouses, IgAN mouses were marked with mild mesangial expansion and cellular proliferation in the mesangial area. Immunohistochemistry showed that the expression of SUMO1 was upregulated in the mesangial area of IgAN mouse model (**Figure 1C**).

Effects of SUMOylation Inhibitors on HMCs' Autophagy Stimulated With AIgA1

The previous study found that mTOR mediated autophagy inhibition, which result in mesangial cell proliferation in IgAN (5). We investigated the protein SUMOylation and autophagy in cultured HMCs treated with aIgA1 and SUMOylation inhibitor GA. The western blotting (WB) showed that the levels of autophagy-related protein LC3 in mesangial cells were significantly lower in the IgA group ($p < 0.01$) as compared to the levels in the control group; however, it was increased

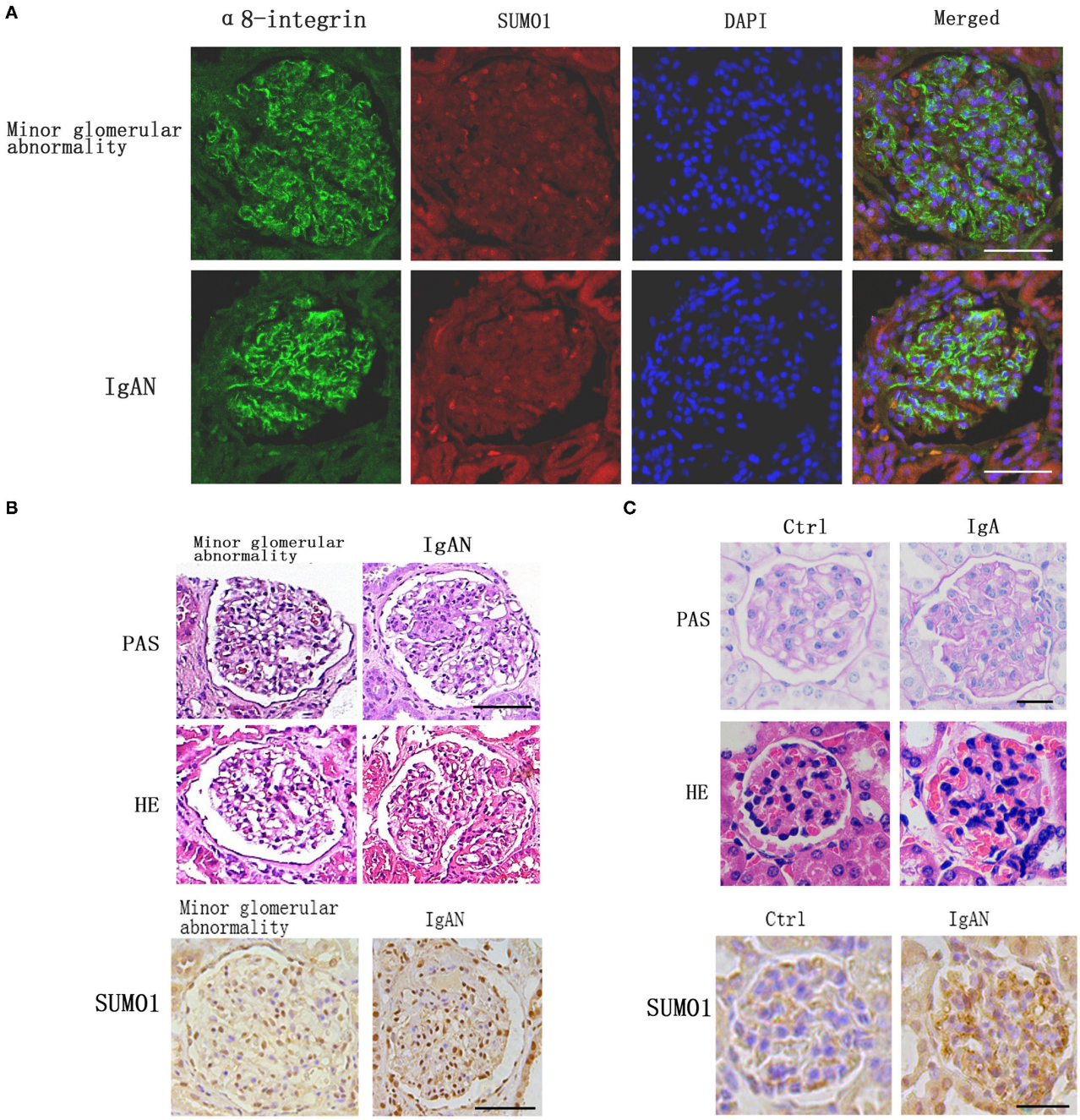

FIGURE 1 | Comparison of SUMO1 expression between IgA nephropathy kidney tissues and adjacent tissues. **(A)** Immunofluorescence staining showed the intraglomerular localization of SUMO1, scale bar 100 μm. **(B)** PAS and HE staining of human kidney tissue. Immunohistochemistry staining for SUMO1 indicated the expression of SUMO1 were up-regulated in IgAN patients group than the minor glomerular abnormality control group, scale bar 100 μm ($n = 10$ per group). **(C)** PAS and HE staining of mouse model kidney tissue. Immunohistochemistry staining for SUMO1 showed the expression of SUMO1 were up-regulated in the mesangial area of IgAN mouse model, scale bar 20 μm ($n = 11$ per group).

in the IgA+GA group compared to the IgA group ($p < 0.01$). The expression of p62 was upregulated in IgA group than control group ($p < 0.05$), and it was downregulated in the IgA+GA group compared to the IgA group ($p < 0.01$) (**Figure 2A**). Autophagy-regulated protein p53 was examined. It was increased significantly in the aIgA1 group ($p < 0.01$). Reductions were observed in the expression of p53 ($p < 0.01$) in aIgA1 and GA treatment groups compared to the aIgA1 group (**Figure 2B**). Real-time PCR showed that the expression of p53 mRNA was upregulated in aIgA1 group

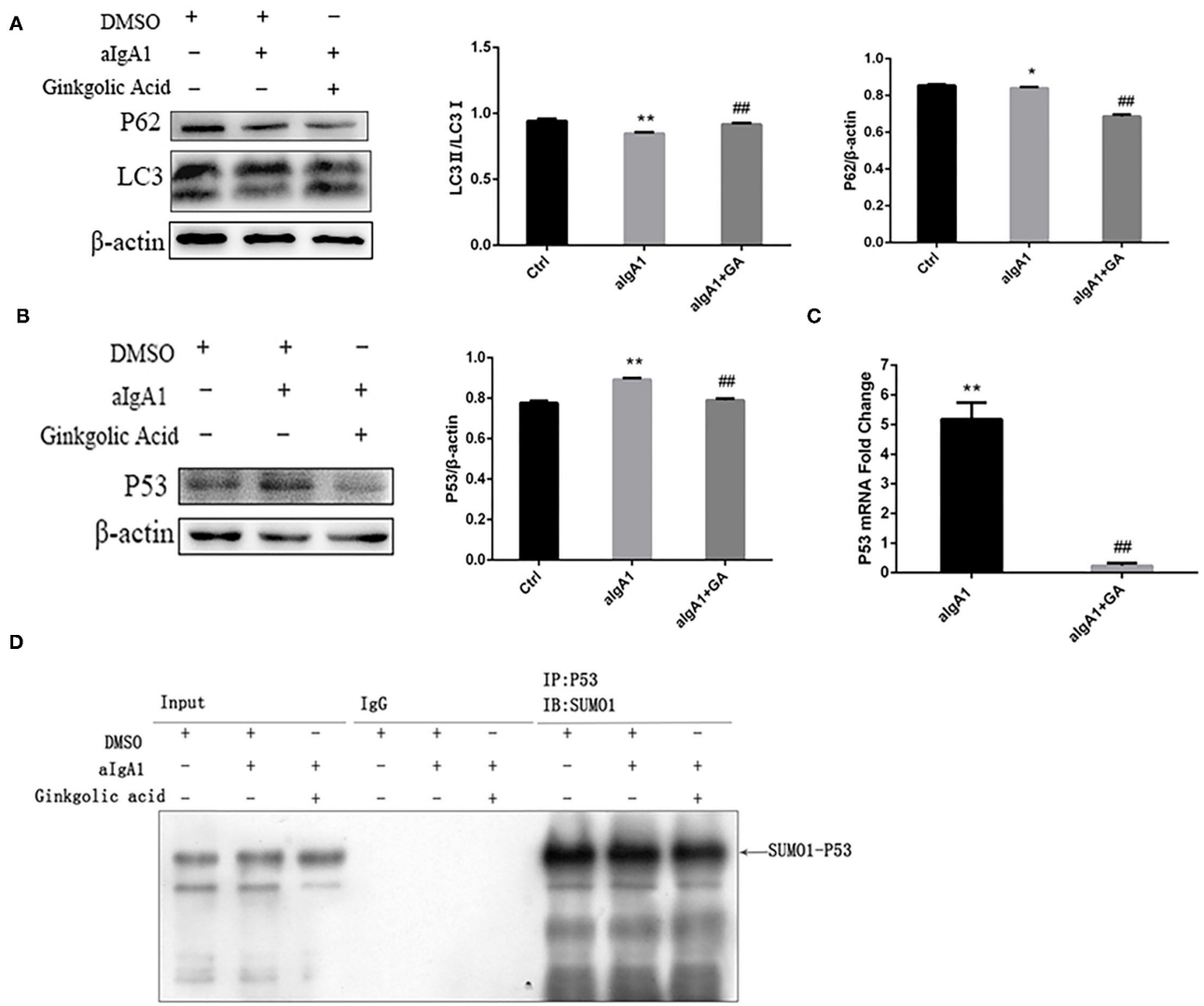

FIGURE 2 | Effects of SUMOylation inhibitors on human mesangial cells' autophagy stimulated by aIgA1. **(A,B)** Western blot analysis for LC3II/I, p62, and p53 in mesangial cells. Cells were treated with aIgA1 and Ginkgolic Acid, and then the whole cells lysates were collected for western bolt analysis. **(C)** Real-time PCR analysis of p53 mRNA. **(D)** Immunoprecipitation for SUMO1-p53 in mesangial cells. Data were presented as mean ± SD ($n = 10$). *$p < 0.05$ vs. control, **$p < 0.01$ vs. control, ##$p < 0.01$ vs. aIgA1 group.

($p < 0.01$). In aIgA1 and GA-treated cells, the expression of p53 mRNA was downregulated (**Figure 2C**). These results concurred with the results of protein expression. We also tested the expression of SUMO1-p53 by immunoprecipitation. In aIgA1 group, the expression of SUMO1-p53 was upregulated (**Figure 2D**). Inhibited SUMOylation could downregulate the expression of SUMO1-p53.

Effects of SUMOylation Inhibitors on HMCs' Proliferation Induced by AIgA1

We examined the proliferation of cells in cultured HMCs stimulated with aIgA1. CCK8 showed that mesangial cell proliferation was observed among groups stimulated with aIgA1. Inhibited SUMOylation with GA could lessen mesangial cell proliferation compared with aIgA1 group ($p < 0.05$) (**Figure 3A**). The expression of cell cycle protein cyclin D1 (CD1) was examined by WB. Cyclin D1 ($p < 0.05$) was increased significantly in the aIgA1 group. Reductions were observed in the expression of cyclin D1 ($p < 0.01$) in aIgA1 and GA treatment groups compared to the aIgA1 group (**Figure 3B**). We further examined cell cycle of HMCs treated with aIgA1 and SUMOylation inhibitors by flow cytometry. In aIgA1 group, the S phase of HMC advanced and the period of cell cycle shortened after stimulated with aIgA1. In the aIgA1+GA group, inhibited SUMOylation could recede S phase and extend the period of cell cycle (**Figure 3C**).

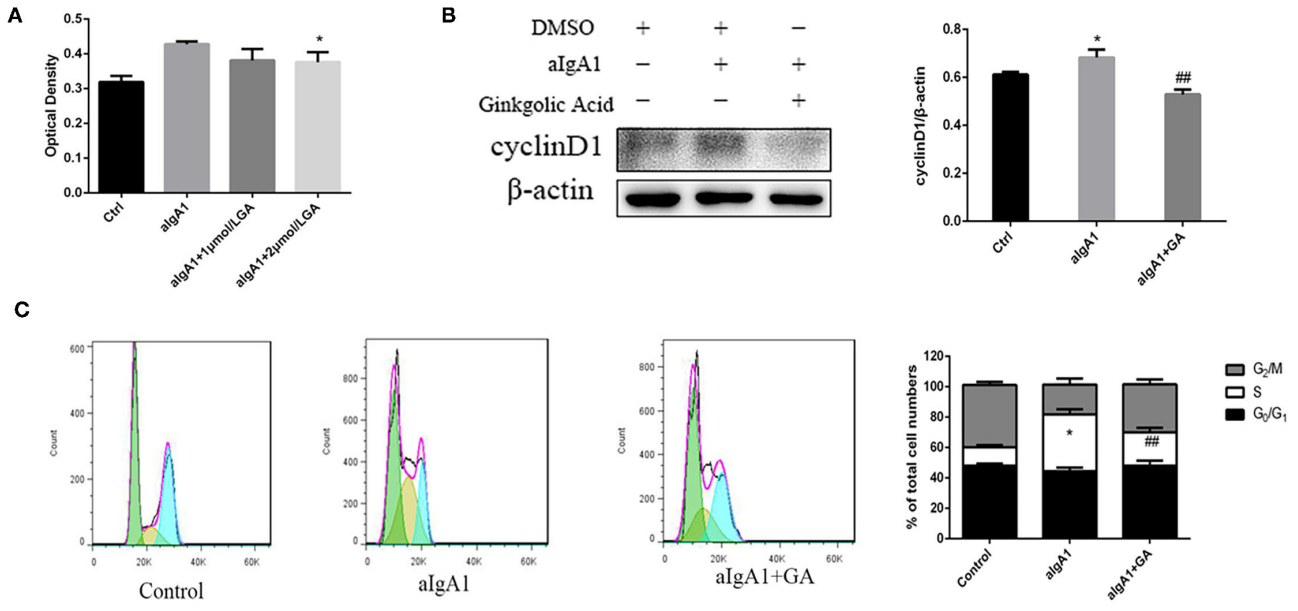

FIGURE 3 | Effects of SUMOylation inhibitors on human mesangial cells' proliferation induced by aIgA1. **(A)** CCK8 analysis of mesangial cells proliferation among groups stimulated with aIgA1 and GA. **(B)** Western blot analysis for Cyclin D1. **(C)** Cell cycle of mesangial cells was analysised by Flow cytometry. Cells were treated with aIgA1 and Ginkgolic Acid. Data were presented as mean ± SD ($n = 10$). $^{*}p < 0.05$ vs. control, $^{\#\#}p < 0.01$ vs. aIgA1 group.

Blockading SUMO1 in Mesangial Cells Suppresses Cell Autophagy Stimulated With AIgA1

To explore the effect of SUMO1 protein on autophagy and cell cycle of HMC, we further transfected HMCs by silence SUMO1 plasmid than stimulated with aIgA1. Silencing SUMO1 made the expression of SUMO1 proteins ($p < 0.01$) and downregulated mRNA ($p < 0.05$) (**Figure 4A**). The autophagy-related protein LC3 ($p < 0.01$) was upregulated in the silencing SUMO1 with aIgA1-stimulated group, whereas the expression of p62 ($p < 0.01$) was downregulated compared to the aIgA1 group (**Figure 4B**). WB of mesangial cells revealed that p53 ($p < 0.01$) was significantly downregulated in the shSUMO1+aIgA1 group than in the shControl+aIgA1 group (**Figure 4C**). We examined the expression of SUMO1-p53 by immunoprecipitation. SUMO1-p53 was downregulated in the shSUMO1+aIgA1 group (**Figure 4D**).

Blockading SUMO1 in Mesangial Cells Suppresses Cell Proliferation and Extends Cell Cycle Stimulated With AIgA1

We then examined the proliferation of mesangial cells by CCK8. In the group that cells transfected with control plasmids and stimulated with aIgA1, cell proliferation was promoted significantly ($p < 0.01$). In the group shSUMO1+aIgA1, cell proliferation was lessened than the shControl+aIgA1 group ($p < 0.01$) (**Figure 5A**). WB revealed that cyclin D1 was downregulated in the shSUMO1+aIgA1 group than in the shControl+aIgA1 group (**Figure 5B**). The cell cycle was tested by flow cytometry. In the shSUMO1+aIgA1 group, silencing SUMO1 could recede S phase and extend the period of cell cycle compared to shControl+aIgA1 group (**Figure 5C**).

DISCUSSION

During the last decade, SUMOylation has been recognized as an important post-translational modification. SUMOylation has been reported to regulate protein subcellular localization, protein-DNA binding, protein–protein interactions, transcriptional regulation, DNA repair, and genome organization. SUMOylation also plays a critical role in autophagy progression. Some targets of SUMOylation implicated in the regulation of autophagy have been identified. One potential target of SUMOylation is the acetyltransferase Tip60/KAT5. Naidu et al. found that SUMOylation of Tip60 may help activate autophagy (22). Another potential target of SUMOylation in regulation of autophagy is PI3KC3/Vps34. SUMOylated Vps34 increases the activity of Vps34 to stimulate autophagy (18, 19). However, given the complexity of autophagy process, the effect of SUMOylation needed more study to identify. Moreover, SUMOylation is known to affect cellular functions in a wild range of disease, which includes cancer, type I diabetes, Crohn's disease, podocyte lesion, and so on (23–26). In kidney disease, SUMOylation determines turnover and localization of nephrin at the plasma membrane (26). Lingyu Wang et al. found that podocytes protect glomerular endothelial cells from hypoxic injury *via* deSUMOylation of HIF-1α (27). In AKI, SUMOylation plays a cytoprotective role (28). In our study, we found in IgAN group and IgA mouse model, the expression of SUMO1 is upregulated in renal cortex. The SUMOylation may

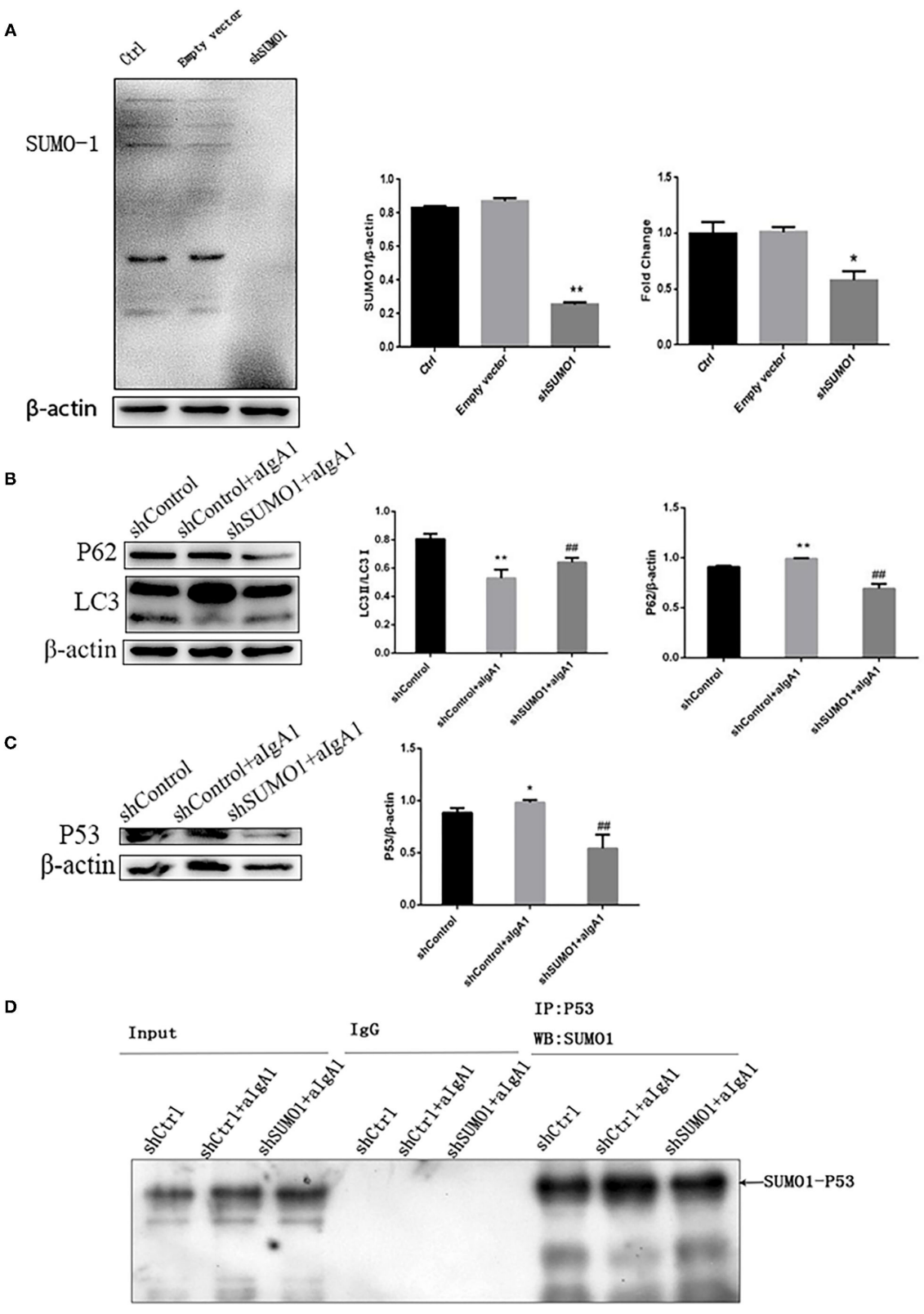

FIGURE 4 | Blockading SUMO1 in mesangial cells suppresses cell autophagy stimulated by aIgA1. **(A)** Western blot analysis and Real-time PCR analysis for SUMO1 in mesangial cells transfected by silencing SUMO1 plasmid. B Western blot analysis for LC3II/I, p62, and p53 in mesangial cells transfected by silencing SUMO1 plasmid. **(C)** Western blot analysis for p53. **(D)** Immunoprecipitation for SUMO1-p53 in mesangial cells. Data were presented as mean ± SD ($n = 10$). $^{*}p < 0.05$ vs. control, $^{**}p < 0.01$ vs. control, $^{\#\#}p < 0.01$ vs. shControl+aIgA1 group.

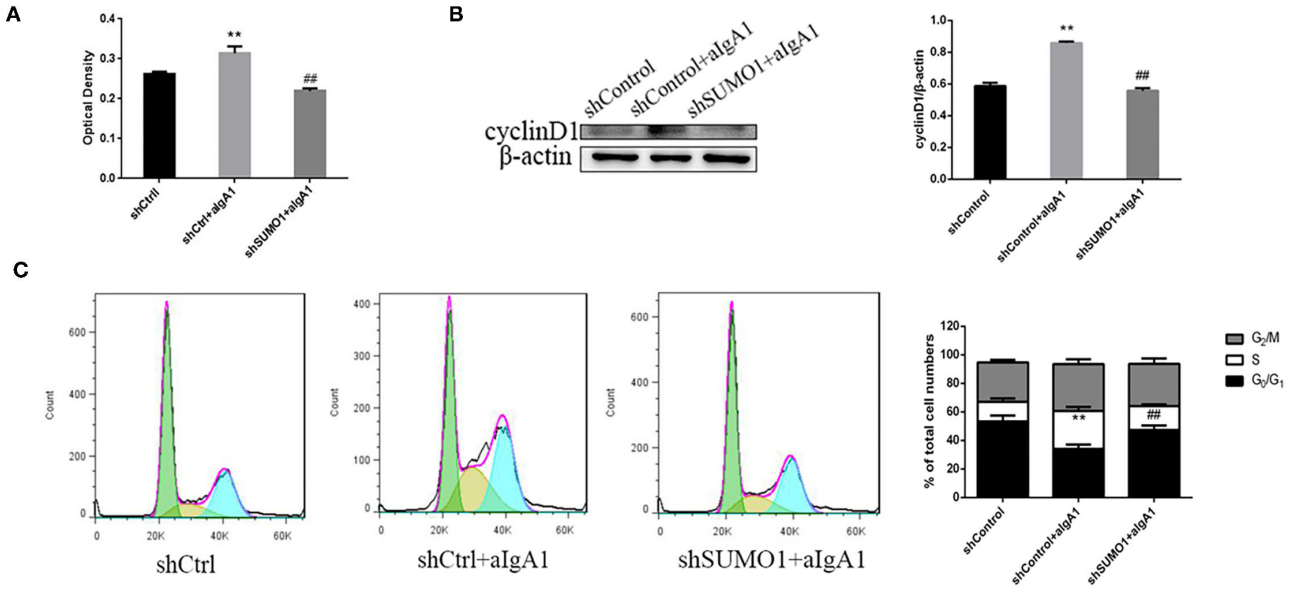

FIGURE 5 | Blockading SUMO1 in mesangial cells suppresses cell proliferation and extends cell cycle stimulated by algA1. **(A)** CCK8 analysis of mesangial cells proliferation among groups transfected with control or shSUMO1 plasmids and stimulated by algA1. **(B)** Western blot analysis for Cyclin D1. **(C)** Cell cycle of mesangial cells was analysised by Flow cytometry. Cells were transfected with control or shSUMO1 plasmids and stimulated by aIgA1. Data were presented as mean ± SD ($n = 10$). $^{**}p < 0.01$ vs. shControl, $\#\#p < 0.01$ vs. shControl+aIgA1 group.

trigger mesangial cell proliferation in IgAN. In the study of human mesangial, the expression of SUMO1 was upregulated. Inhibited SUMOylation or SUMO1 could lessen mesangial cell proliferation.

Impaired or deficient autophagy is believed to contribute to proliferative diseases in the previous studies (5). Some factors altered intracellular signaling pathways and autophagy activities under several pathological conditions. In proliferative diseases especially cancer, autophagy modulation is considered to be a double-edged sword. In cancer, autophagy can be neutral, tumor-suppressive, or tumor-promoting in different contexts (29). According to the growing literature, autophagy inhibits malignant transformation (30). Autophagy could buffer metabolic stress in tumor cells (31, 32). Impaired or deficient autophagy is believed to contribute to kidney disease as described in the previous studies that focused on the role of autophagy in kidney disease, especially in acute kidney injury and renal fibrosis (33, 34). In acute kidney injury, autophagy has shown protective properties in ischemic and cisplatin-induced AKI models (33). But in renal fibrosis, the role of autophagy is dual (34). Maybe, that the autophagy can switch roles like in cancer, which depends on the stage of the disease. In IgAN, the previous study showed that autophagy inhibition may result in mesangial cell proliferation and renal lesions (5). In our study, we found that the autophagy-related protein LC3II/LC3 was upregulated, and p62 was downregulated. The aIgA1 could repress the mesangial cell autophagy, shorten the period of cell cycle, and trigger the mesangial cell proliferation.

Inhibited SUMOylation could inhibit SUMO1-p53 and activate autophagy. The key tumor suppressor protein p53 has a number of tasks, which include regulating cell cycle and autophagy (35). P53 controls the process of autophagy by its own activation or deactivation. Recent studies suggest that by different intracellular positioning, p53 plays different roles in autophagy regulation: p53 facilitates autophagy when positioned in the cell nuclei because it can transactivate the two subunits of AMPK as well as TSC2. Meanwhile, p14ARF that exists in the cell nuclei can easily bind with ubiquitin hydrolase MDM2 of p53 to effectively prevent p53 from the degradation caused by MDM2, thus preserving the role of p53 in the nucleus (36); when positioned in the cytoplasm, p53 inhibits autophagy in three ways: activating autophagy inhibitor mTOR, inhibiting the effect of AMPK, and exerting a direct effect (37). As a target of SUMO1, the SUMOylation could mediate p53 transactivation and apoptosis (38, 39). The SUMO1-p53 may play an important role in regulating autophagy. In IgAN, p53 is upregulated in intrinsic renal cells (17). In our study, we found that aIgA1 could repress the mesangial cell autophagy, shorten the period of cell cycle, and trigger the mesangial cell proliferation. Inhibited or silenced SUMOylation could inhibit SUMO1-p53 and activate autophagy. The SUMOylation of p53 in cytoplasm may help to inhibit autophagy.

In conclusion, the expression of SUMO1 and SUMO1-p53 was increased and autophagy was inhibited, and also, the expression of cyclin D1 was increased in aIgA1-stimulated HMC. After GA treatment, SUMO1 and SUMO1-p53 were decreased, and autophagy was increased, while the cell proliferation was

lessened. Our findings suggested that overexpression of SUMO1 mediated autophagy inhibition, which may result in mesangial cell proliferation in IgAN, and SUMO1-p53 may play a role in this process.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding authors.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Medical Ethics Review Committee of The Second Xiangya Hospital of Central South University. The patients/participants provided their written informed consent to participate in this study. The animal study was reviewed and approved by Medical Ethics Review Committee of The Second Xiangya Hospital of Central South University.

AUTHOR CONTRIBUTIONS

XTan and HL performed study concept and design. XTan, DL, and YL performed development of methodology and writing. XTang and MX review and revision of the paper. LH provided analysis and statistical analysis. GC and XZ provided technical and material support. All authors read and approved the final paper.

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New Insights Into an Overlooked Entity: Long-Term Outcomes of Membranous Lupus Nephritis From a Single Institution Inception Cohort

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Introduction: Pure membranous lupus nephritis (MLN) accounts for 10–20% of total cases of lupus nephritis and is generally associated with a better patient and renal survival compared to proliferative classes. Studies of MLN are limited by small sample size and heterogeneity of included populations since patients with pure MLN and those with mixed classes are usually examined together.

Aim of the Study: To describe clinical and laboratory characteristics of patients with pure MLN, therapeutic regimens, response to treatment, renal relapses, and their long-term renal survival and to define prognostic factors of remission and relapse.

Methods: We retrospectively studied an inception cohort of 27 patients with histologically proven pure MLN. Clinical, laboratory and therapeutical parameters were recorded at diagnosis, at different time points (3–6–9–12–18–24–36–72 months) during the course of the disease, at time of renal flare, and at last follow up visit.

Results: 48.1% (13/27) of patients were treated with mycophenolic acid (MPA), 29.6% (8/27) with cyclophosphamide (CYC), and 3.7% (1/27) with cyclosporine (all in combination with corticosteroids). Five patients (18.5%) did not receive any immunosuppressive treatment. Mean duration of treatment was 4.7 ± 2.3 years. Median time to complete remission was 9 months (IQR = 7) and median time to partial remission was 4 months (IQR = 4). No clinical or laboratory parameter was found to be significantly associated with time to remission. Time to remission was not significantly affected by either of the two treatment regimens (CYC and MPA) ($p = 0.43$). Renal flare was observed in 6 (22%) of the 27 patients in a median time of 51 months (IQR = 63). Proteinuria >1 g/24 h at 1 year significantly correlated with risk of flare (OR 20, $p = 0.02$). After a median follow up period of 77 months, all patients had an eGFR > 60 ml/min/1.73 m^2 (mean eGFR 100 ± 32 ml/min/1.73 m^2).

Conclusions: In a small cohort of patients with pure MLN, long-term renal survival was very good. With the limitation of the small sample size, we could not find any

baseline clinical, biochemical or therapeutic factor that could predict time to remission. Proteinuria > 1 g/24 h at 1 year should be further examined in larger cohorts as a possible predictor of flare.

Keywords: membranous, lupus nephritis, remission, flare, outcomes

INTRODUCTION

Renal involvement in systemic lupus erythematosus (SLE) may occur in 25–60% of patients during the course of the disease, and frequently at the time of first diagnosis of SLE (1–3). Lupus nephritis (LN) has long been considered as a major cause of morbidity and mortality in lupus patients (4).

Despite the knowledge gained in regard to the pathogenesis, clinical presentation and natural history of LN and the advances in treatment over the past decades, about 10–30% of LN patients will develop end stage renal disease (ESRD) (5, 6). Renal prognosis differs by race and ethnicity with African-Americans and Hispanics having worse renal outcomes than Asians and Caucasians (1–3). Lupus nephritis histological class is a major determinant of renal survival with up to 30% of patients with proliferative classes progressing to ESRD within 10 years compared to only 10% of those with membranous LN (7).

Pure membranous LN (MLN) accounts for 10–20% of total cases of LN and, although a better renal and patient prognosis compared to proliferative classes has been recognized, the risk of ESRD is not negligible ranging from 0 to 23% at 10 years in previous studies. Furthermore, MLN often presents with nephrotic syndrome which may be associated with thrombotic and infectious complications that negatively affect patients' morbidity and mortality (8–13).

Studies of membranous lupus nephritis (MLN) are often limited by the small samples, non- inception cohorts, the heterogeneity of examined populations and indirect evidence since composite data from pure MLN, and mixed MLN with proliferative classes are often presented. Also, there is limited evidence on prognostic factors of disease remission, relapse and long-term renal survival of patients with pure MLN and on optimal treatments.

The aim of the present study is: (a) to describe clinical and laboratory characteristics of patients with pure MLN, therapeutic approaches, response to treatment, and their long-term renal survival, and (b) to define prognostic factors of remission and flare.

PATIENTS AND METHODS

Study Population

This is an inception cohort study of all patients with pure MLN diagnosed between 2001 and 2016 and followed at our joint academic center (Nephrology and Rheumatology Units) at Laiko Hospital until June 2019. All patients fulfilled the ACR classification criteria for SLE and lupus nephritis diagnosis was confirmed by renal biopsy. Pure MLN (class V) was classified according to the International Society of Nephrology/ Renal Pathology Society (ISN/RPS) 2003 lupus nephritis classification. Biopsies performed before 2003 were reassessed based on ISN/RPS 2003 classification system.

Data Collection

Medical charts of patients were retrospectively reviewed and clinical, laboratory, and therapeutical parameters were recorded at the time of LN diagnosis and at 6–12–18–24–36–72 months after MLN diagnosis, at the time of renal flare (with or without a repeat biopsy) and at last follow-up visit. Patients with mixed MLN and <6 months of follow-up were excluded.

Data collected included demographic parameters, time from SLE diagnosis to LN onset, disease activity (expressed by Systemic Lupus Erythematosus Disease Activity Index 2000, SLEDAI-2K score) (14), anti-ds DNA titers, C3 and C4 levels, serum urea (Ur), creatinine (Cr) and albumin, eGFR (based on the CKD-EPI formula), 24-h proteinuria, urine sediment, renal biopsy histological parameters, and immunosuppressive treatment.

The study was approved by the Institutional Review Board (IRB) of Laiko General Hospital of Athens and Medical School of National Kapodistrian University of Athens. Due to the retrospective nature of the study, an informed consent was not required.

Definitions

Remission and flare were defined according to the EULAR/ERA-EDTA (15) and the KDIGO recommendations (16). Active urine sediment was defined as the presence of >5 RBCs/hpf or ≥1 red cell casts. Complete remission (CR) was defined as proteinuria <500 mg/24 h and serum creatinine reduction within 10% from baseline. Partial remission (PR) was defined as ≥50% reduction in proteinuria to subnephrotic levels and serum creatinine within 10% from baseline. Nephritic flare was defined as an increase in glomerular haematuria by ≥10 RBCs/hpf with or without a decrease in GFR by ≥10%, irrespective of changes in proteinuria. Nephrotic flare was defined as reproducible doubling of proteinuria to >1,000 mg/24 h if complete response had been previously achieved or as reproducible doubling of proteinuria to ≥2,000 mg/24 h if partial response has been previously achieved. "Early" MLN was defined as onset of MLN < 1 year from SLE diagnosis. "Late" MLN was defined as onset of MLN > 1 year from SLE diagnosis.

Statistical Analysis

Continuous variables were expressed as the mean value and standard deviation or median value and interquartile range (IQR), whereas categorical variables as frequencies and percentages. To investigate the differences between baseline demographic, clinical, and laboratory variables between patients with different therapeutical schemes, the *t*-test and Mann–Whitney *U*-test for independent samples for continuous

variables and the χ^2 and Fisher exact test for categorical variables were applied. Univariate logistic regression analyses were performed to estimate the prognostic effect of various variables on the risk of renal flare and Cox regression analyses for investigating the association between the time to remission (either partial or complete) of patients with MLN and their clinical characteristics. Variables that were found to be significant (significance was set at $\alpha = 0.05$) in the univariate analyses, as well as variables that showed to have a predictive role even though not strictly significant ($p < 0.10$), were included in the multivariate models. Since the number of flares recorded was small (only 6), multivariate logistic regression analysis could not be performed because it would be vulnerable to errors. The estimated odds ratios (ORs) and hazard ratios (HRs) of both the univariate and multivariate models, as well as the related *p*-values, are presented. Data were analyzed using Stata 13.0 software (Stata Corporation, College Station, TX). All tests proceeded as 2 tailed.

RESULTS

Baseline Demographic, Clinical and Biochemical Parameters

The baseline demographic, clinical, laboratory, and histological parameters are shown in **Table 1**.

Of note, all patients of the cohort were Caucasians.

Treatment Regimens

All patients received ACE inhibitor or ARB. Thirteen patients (48.1%) were treated with mycophenolic acid (MPA), 8 (29.6%) with cyclophosphamide (CYC) and 1 (3.7%) with cyclosporine (all in combination with corticosteroids) (**Table 1**). Five patients (18.5%) did not receive any immunosuppressive treatment because of low grade proteinuria, according to the existing at that time recommendations. Eight (29.6%) patients were on hydroxychloroquine (HCQ) treatment at the time of LN diagnosis (**Table 1**). However, the majority of patients (22/27, 81.5%) received HCQ at some point during the course of their disease.

Patients treated with MPA and those treated with CYC differed significantly only in eGFR levels at baseline (**Table 2**). eGFR at baseline was lower in the CYC group (mean ± SD 74.6 ± 40.6) than in the MPA group (mean ± SD 110 ± 28.2) ($p = 0.02$).

Mean duration of treatment was 4.7 ± 2.3 years and did not differ significantly between the two treatment groups.

When we divided patients into those with "early" (<1 year since SLE diagnosis) onset of MLN and those with "late" (>1 year since SLE diagnosis) onset of MLN, we found that the two groups differed significantly in regard to baseline proteinuria. "Early" MLN patients had a median baseline proteinuria of 5.5 g/d (IQR = 4.3) vs. 2.7 g/d (IQR = 2.1) in "late" MLN ($p = 0.03$) (**Table 3**).

Remission Rates and Prognostic Factors of Remission

At 6 months, 77% of the total cohort achieved remission (37% CR, 40% PR). At 12 months, 89% were in remission (70% CR, 19% PR) and at the end of follow-up (median 77 months), all patients were in remission (89% CR, 11%PR) (**Figure 1**).

TABLE 1 | Baseline demographic, clinical, and laboratory characteristics and treatment regimens.

Baseline characteristics	Mean ± SD, median/IQR, *N*/%
Age (year) mean ± SD	47 ± 12
Sex (M–F) *N*/%	5/19–22/81
Duration of SLE (months) median/IQR	4/72
SLEDAI score mean ± SD	10.5 ± 4
Low C3 *N*/%	18/69.2
Low C4 *N*/%	16/61.5
Positive anti-dsDNA *N*/%	19/76
Proteinuria (g/24 h) mean ± SD	4.9 ± 3.6
Proteinuria > 3 g/d *N*/%	18/67
Proteinuria 1–3 g/d *N*/%	6/22
Proteinuria < 1 g/d *N*/%	3/11
Active urine sediment *N*/%	19/70
Hypertension *N*/%	4/14.8
Serum albumin (g/dl) mean ± SD	3.1 ± 0.8
Serum Cr (mg/dl) mean ± SD	1 ± 1
eGFR (ml/min/1.73 m^2)mean ± SD	111 ± 34
eGFR > 60 N/%	24/89
eGFR 30–60 *N*/%	1/3.7
eGFR < 30 *N*/%	2/7.3
Induction treatment	
Mycophenolic acid *N*/%	13/48.1
Cyclophosphamide *N*/%	8/29.6
Cyclosporine *N*/%	1/3.7
None *N*/%	5/18.5
Maintenance treatment	
Mycophenolic acid *N*/%	18/66.7
Cyclophosphamide *N*/%	2/7.4
Cyclosporine *N*/%	1/3.7
Azathioprine *N*/%	1/3.7
None *N*/%	5/18.5
Hydroxychloroquine	
Yes *N*/%	8/29.6
No *N*/%	19/70.4
Follow up (months) median/IQR	*77/64*

eGFR, estimated glomerular filtration rate using the CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; anti-ds DNA, antibodies against double stranded DNA.

The median time to complete remission was 9 months (IQR = 7) and the median time to partial remission was 4 months (IQR = 4). Median time to remission did not differ significantly between patients treated with MPA acid and those treated with CYC. Median time to complete remission in MPA group was 8 months (IQR = 6) vs. 6 months (IQR = 18) in CYC group ($p = 0.84$; **Figure 2**), median time to partial remission was 3 months (IQR = 2) in both treatment arms ($p = 0.48$), and median time to either complete or partial remission was 3 months (IQR = 2) in both groups ($p = 0.48$).

TABLE 2 | Comparison of baseline characteristics between the two treatment groups (mycophenolic acid vs. cyclophosphamide).

Baseline characteristics	Treatment with mycophenolic acid (*N* = 13)	Treatment with cyclophosphamide (*N* = 8)	*p*-value
Age (year) mean ± SD	45 ± 15	50 ± 8	0.5
Sex (M–F) *N*/%	3/23–10/77	1/12.5–7/87.5	1
Duration of SLE (months) median/IQR	38/108	8/12	0.41
SLEDAI score mean ± SD	10.2/3.8	10.8/4.3	0.7
Low C3 *N*/%	8/67	6/75	1
Low C4 *N*/%	7/58	6/75	1
Positive anti-dsDNA *N*/%	8/66.7	6/75	1
Proteinuria (g/24 h) mean ± SD	4.7 ± 2.7	6.5 ± 5.2	0.33
Proteinuria > 3 g/d *N*/%	9/69	6/75	1
Proteinuria 1–3 g/d *N*/%	3/23	1/12.5	
Proteinuria < 1 g/d *N*/%	1/8	1/12.5	
Active urine sediment *N*/%	11/84.6	4/50	0.14
Hypertension *N*/%	1/7.6	2/25	0.53
Serum albumin (g/dl) mean ± SD	3.1 ± 0.8	2.8 ± 0.7	0.49
Serum Cr (mg/dl) mean ± SD	0.7 ± 0.24	1.7 ± 1.6	0.056
eGFR (ml/min/1.73 m^2) mean ± SD	110 ± 28.2	74.6 ± 40.6	**0.02**
eGFR > 60 *N*/%	12/92	6/75	0.13
eGFR 30–60 *N*/%	1/8	–	
eGFR < 30 *N*/%	–	2/25	
Duration of treatment (months) mean ± SD	4.4 ± 2.8	5.2 ± 3	0.56

eGFR, estimated glomerular filtration rate using the CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; anti-ds DNA, antibodies against double stranded DNA.
Bold values are those with statistical significance ($p < 0.05$).

TABLE 3 | Comparison of baseline characteristics between "early" (<1 year) and "late" (>1 year) LMN patients.

Baseline characteristics	Early MLN (*N* = 14)	Late MLN (*N* = 13)	*p*-value
Age (year) mean ± SD	44 ± 11	50 ± 11	0.11
Sex (M–F) N/%	2/14–12/86	3/23–10/77	0.6
Duration of SLE (months) median/IQR	0/1	72/84	<0.01
SLEDAI score median/IQR	11.7/3.9	9.3/3.6	0.08
Low C3 *N*/%	11/78	7/58	0.4
Low C4 *N*/%	9/64	7/58	1
Positive dsDNA *N*/%	11/78	8/72	1
Proteinuria (g/24 h) median/IQR	5.5/4.3	2.7/2.1	**0.03**
Proteinuria > 3 g/d *N*/%	12/86	6/47	0.08
Proteinuria 1–3 g/d *N*/%	1/7	5/38	
Proteinuria <1 g/d *N*/%	1/7	2/15	
Active urine sediment *N*/%	10/72	9/69	1
Hypertension *N*/%	3/21	1/7	0.6
Serum albumin (g/dl) mean ± SD	2.9 ± 0.8	3.4 ± 0.6	0.23
Serum Cr (mg/dl) mean ± SD	0.6 ± 0.6	0.7 ± 0.2	0.63
eGFR (ml/min/1.73 m^2) mean ± SD	96.1 ± 41.3	103 ± 25	0.7
eGFR > 60 *N*/%	12/86	12/92	0.4
eGFR 30–60 *N*/%	–	1/8	
eGFR < 30 *N*/%	2/14	–	
Duration of treatment (years) mean ± SD	5 ± 2.7	3.7 ± 1.9	0.31
Induction treatment			
Mycophenolic acid *N*/%	7/50	6/46	1
Cyclophosphamide *N*/%	4/28	4/30	1
Cyclosoprine N/%	–	2/15	0.2

eGFR, estimated glomerular filtration rate using the CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; anti-ds DNA, antibodies against double stranded DNA.
Bold values are those with statistical significance ($p < 0.05$).

Time to complete remission differed significantly between patients with "early" MLN (median 6.5 months, IQR = 8) and those with "late" MLN (median 11 months, IQR = 8; $p = 0.05$; **Figure 3**).

In Cox regression analysis no clinical or laboratory parameter was found to be significantly associated with time to remission (CR or PR; **Table 4**). Neither of the two treatment regimens (CYC and MPA) correlated to time to remission (HR = 0.69, $p = 0.43$). No significant correlation was found between onset of MLN ("early" vs. "late") and remission time (HR = 0.61, $p = 0.22$).

Renal Flares and Prognostic Factors of Flare

Renal flare was observed in 6 (22%) of the 27 patients in a median time of 51 months (IQR = 63; **Figure 4**).

Only one of these flares was nephritic and five were nephrotic. Among the patients who had a flare, 2 (33%) had been treated with CYC, 2 (33%) with MPA, 1 (17%) with cyclosporine while 1 patient (17%) had never received any immunosuppressive treatment. In 3 (50%) of six cases no repeat biopsy was performed. In the other three cases, the repeat biopsy did not reveal a class switch.

In univariate logistic regression analysis, proteinuria>1 g/24 h at 1 year significantly correlated with risk of flare (OR = 20, $p = 0.02$; **Table 5**). eGFR > 60 ml/min/1.73 m^2 at diagnosis, proteinuria 1–3 g/24 h at diagnosis, female sex and treatment with MPA were associated with a lower risk of flare but not in a statistically significant manner. Hypertension and low C3 and C4 levels at diagnosis were associated with increased risk of flare but this correlation wasn't statistically significant. Multivariate analysis was not possible due to small number of events.

Renal and Patient Survival

No patient in our cohort developed ESRD in a median follow up period of 77 months. In fact, all patients at the end of follow up had an eGFR>60 ml/min/1.73 m^2 (mean eGFR 100 ± 32 ml/min/1.73 m^2). Notably, 89% of the patients had an eGFR>60 ml/min/1.73 m^2 at the time of MLN diagnosis.

At the end of follow up, all patients were in remission (89% CR, 11% PR), and mean 24 h proteinuria was 0.12 ± 0.12 g. Ten (37%) of 27 patients did not receive any immunosuppressive

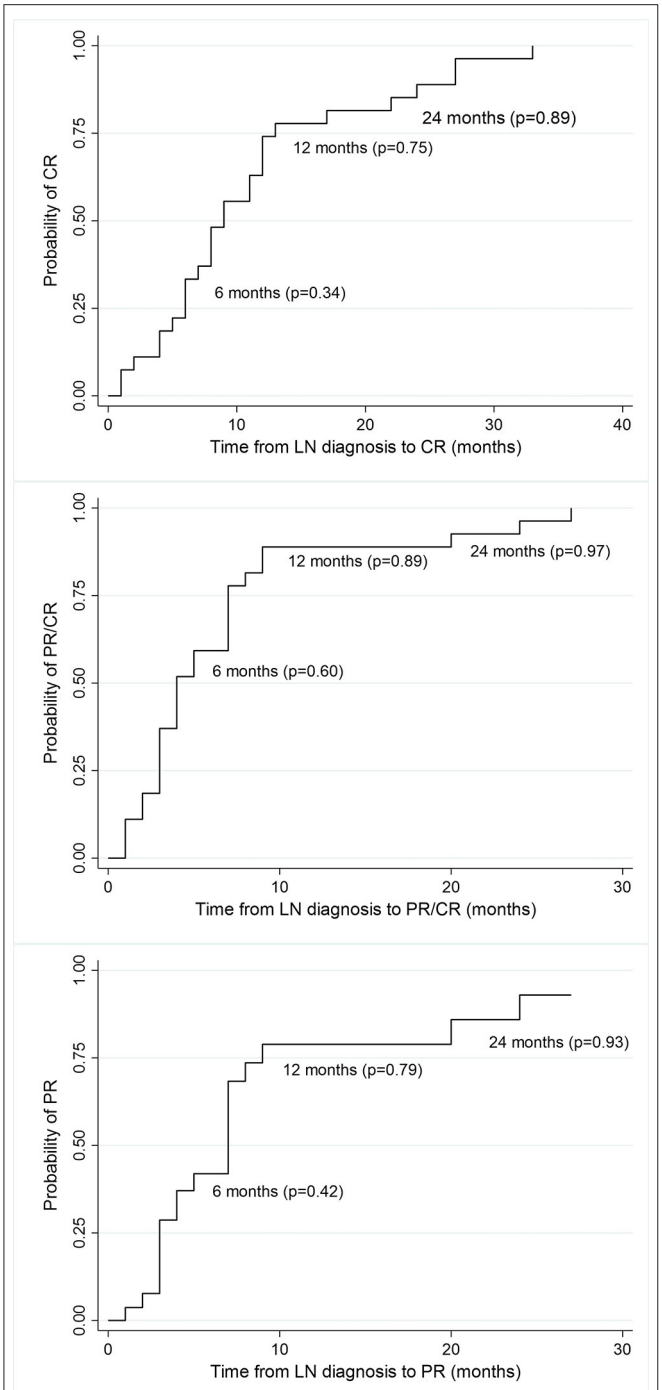

FIGURE 1 | Kaplan-Meier estimates of survival probability of partial and complete remission at different time points from LN diagnosis. CR, complete remission; PR, partial remission; p, probability.

drug, 13 (48%) continued immunosuppressive treatment (10 on MPA, 3 on AZA) and 4 (15%) were on corticosteroids only.

At a median follow up time of 77 months, no death was recorded but there were 10 patients lost to follow up after 3 years (all in remission).

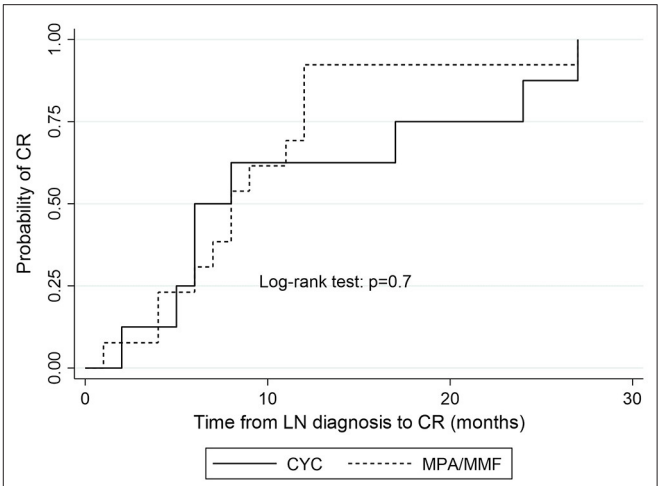

FIGURE 2 | Kaplan-Meier estimates of survival probability of complete remission according to induction treatment. CYC, cyclophosphamide; MPA, mycophenolic acid; CR, complete remission.

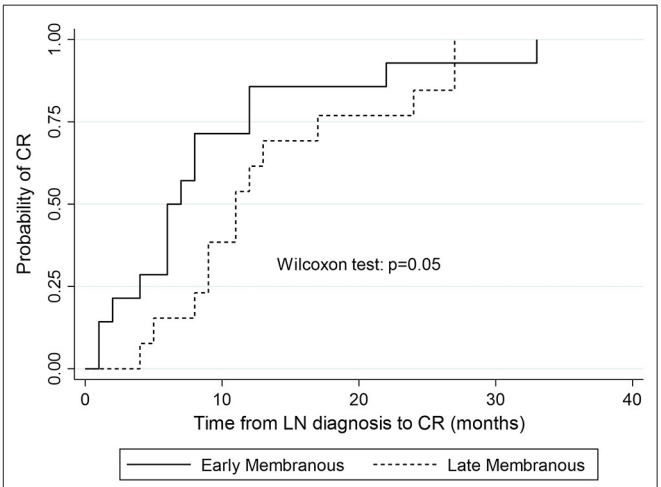

FIGURE 3 | Kaplan-Meier estimates of survival probability of complete remission in "early" vs. "late" MLN.

Adverse Events

No thrombotic or cardiovascular event occurred. One episode of herpes zoster and one episode of HBV reactivation were successfully managed with antiviral therapy and temporary reduction of immunosuppression.

DISCUSSION

Knowledge gained in the field of pure MLN shows a favorable renal prognosis (compared to proliferative and mixed forms of LN) and underscores the need of treatment regimens consisting of a combination of corticosteroids and an immunosuppressive agent, even in patients with subnephrotic levels of proteinuria. The optimal immunosuppressant agent as well as the optimal duration of treatment are not yet fully elucidated. Achievement

TABLE 4 | Correlations of clinical, laboratory, and treatment parameters with time to remission (either partial or complete).

Variables	Univariate models		Multivariate model	
	HR	95% CIs (*p*-value)	HR	95% CIs (*p*-value)
eGFR at diagnosis (ml/min/1.73 m^2)				
<30	Reference Group			
31–60	0.18	0.01, 2.23 (0.18)	0.37	0.02, 5.25 (0.46)
>60	0.22	0.04, 1.06 (0.06)	0.26	0.05, 1.29 (0.1)
Proteinuria at diagnosis (g/day)				
<1	Reference Group			
1–3	1.95	0.37, 10.2 (0.42)		
>3	2.01	0.45, 8.9 (0.35)		
Age (years)	1.01	0.97, 1.04 (0.49)		
Sex				
Male	Reference Group			
Female	0.58	0.21, 1.6 (0.29)		
Diagnosis of SLE to LN (years)	0.98	0.89, 1.07 (0.68)		
Time of LN after SLE diagnosis				
Early (<1 year)	Reference Group			
Late (>1 year)	0.61	0.28, 1.34 (0.22)		
SLEDAI score	1.1	0.98, 1.24 (0.099)		
Induction treatment				
Cyclophosphamide	Reference Group			
Mycophenolic acid	0.69	0.28, 1.71 (0.43)		
Hypertension				
No	Reference Group			
Yes	2.26	0.75, 6.79 (0.144)		
Low C3				
No	Reference Group			
Yes	2.37	0.98, 5.7 (0.054)	2.28	0.45, 11.5 (0.31)
Low C4				
No	Reference Group			
Yes	2.13	0.93, 4.9(0.07)	1.02	0.22, 4.65 (0.97)
Anti-ds-DNA				
Negative	Reference Group			
Positive	0.69	0.93, 4.9 (0.45)		

eGFR, estimated glomerular filtration rate using the CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; anti-ds DNA, antibodies against double stranded DNA.

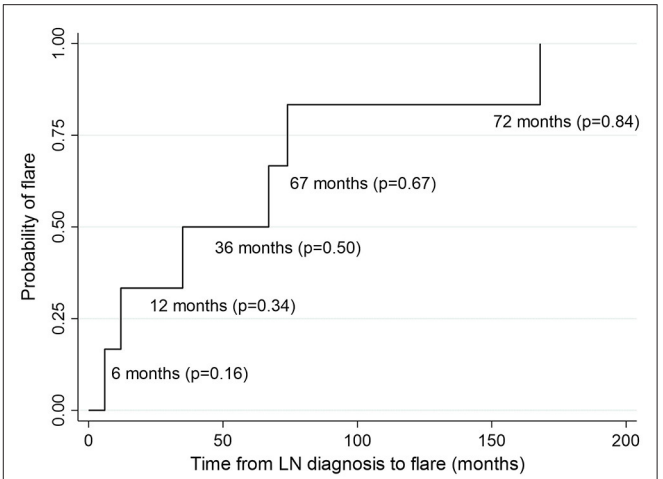

FIGURE 4 | Kaplan-Meier estimates of survival probability of renal flare at different time points from LN diagnosis. P, probability.

of remission, time till remission and flare occurrence have been recognized to affect the long-term renal outcome in LN patients. However, the issue of factors that could predict time to remission and flare occurrence in the subpopulation of patients with pure MLN has not been fully addressed. Time to remission (and not only achievement of remission *per se*) is of great importance in these patients, since longer time to remission exposes them to a greater risk of thrombotic and infectious complications associated with the high levels of proteinuria patients with pure MLN have. We have showed that no baseline clinical or laboratory parameter (not even the level of baseline proteinuria) could serve as a prognostic factor of time to remission and that both major treatment regimens (MPA and CYC) induced remission in similar times. Twelve-month proteinuria has been recognized as a predictor of long-term renal survival in the total cohort of LN patients. Our study suggests that 12-month proteinuria can be also used as a prognostic factor of flare in pure MLN patients. Renal survival of patients with MLN ranges from 96 to 98% at 5 years, 72–100% at 10 years, and reaches 83% at 15 years (7, 17–22). Nevertheless, in certain ethnic groups, such as African Americans, it may be significantly lower (71% at 5 years) (23). In accordance with previous studies, renal survival in our cohort was excellent with all of the patients having an eGFR > 60 ml/min/1.73 m^2 at a median follow up time of 77 months. Even the three patients who had an eGFR < 60 ml/min/1.73 m^2 at diagnosis (2 of them with eGFR<20 ml/min/1.73 m^2) managed to improve their renal function during the first 12 months. Renal injury in these patients can be attributed to hemodynamic changes caused by proteinuria and as the latter resolved with treatment, renal function recovered. The excellent renal survival in our cohort can be attributed to the fact that all patients were Caucasians and the majority had normal renal function at diagnosis. The fact that these patients were very closely followed up at a center highly experienced in the management of LN may have also contributed to the good renal outcomes (24), as it was previously shown (17).

The optimal treatment for MLN has not yet been fully elucidated. There is, however, strong evidence that a combination of corticosteroids and immunosuppressives is superior to steroid monotherapy (25, 26). Azathioprine has been shown to be effective in achieving remission but it was associated with high relapse rates (18, 19). Several studies have reported similar rates of clinical response between MPA and CYC (27–29) while others, including a network meta-analysis, have showed superiority of MMF over CYC (21, 30, 31). Calcineurin inhibitors have also been used to treat pure MLN with similar overall response compared to MMF, CYC or AZA (26, 32–35). CNIs are often associated with a faster resolution of proteinuria (35) but also with a higher relapse

TABLE 5 | Prognostic factors of renal flare.

Variables	Univariate models	
	OR	**95% CIs (*p*-value)**
eGFR at diagnosis (ml/min/1.73 m^2)		
<30		Reference Group
31–60	–	–
>60	0.26	0.01, 4.98 (0.37)
Time to PR (months)	1	0.87, 1.15 (0.94)
Time to CR (months)	1.05	0.94, 1.16 (0.34)
Time to PR/CR (months)	1	0.87, 1.13 (0.94)
Proteinuria at diagnosis (g/day)		
<1		Reference Group
1–3	0.52	0.04, 5.62 (0.59)
>3	–	–
Proteinuria at 12 months (g/day)		
<1		Reference Group
>1	**20**	**1.53, 260 (0.02)**
Age (years)	1	0.93, 1.08 (0.85)
Sex		
Male		Reference Group
Female	0.33	0.04, 2.69 (0.30)
Diagnosis of SLE to LN (years)	0.98	0.96, 1.01 (0.31)
Time of LN after SLE diagnosis		
Early (<1 year)		Reference Group
Late (>1 year)	0.45	0.06, 3.04 (0.41)
SLEDAI score	1.07	0.83, 1.37 (0.57)
Induction treatment		
Cyclophosphamide		Reference Group
Mycophenolic acid	0.54	0.06, 4.91 (0.58)
Hypertension		
No		Reference Group
Yes	1.2	0.10, 14.1 (0.88)
Low C3		
No		Reference Group
Yes	2.69	0.26, 27.8 (0.40)
Low C4		
No		Reference Group
Yes	4.09	0.40, 41.6 (0.23)
Anti-dsDNA		
Negative		Reference Group
Positive	–	– – –

eGFR, estimated glomerular filtration rate using the CKD-EPI formula; SLEDAI, systemic lupus erythematosus disease activity index; anti-ds DNA, antibodies against double stranded DNA; CR, complete remission; PR, partial remission.
Bold values are those with statistical significance ($p < 0.05$).

rate compared to MMF or CYC (32). These observations may, in part, be explained by the fact that CNIs, besides their immunosuppressive actions, affect the intraglomerular pressure and act on the podocytes' cytoskeleton leading to proteinuria reduction (36). In our cohort, 48.1% of patients were treated with MPA, 29.6% with CYC, and only one patient with cyclosporine. The two treatment groups (MPA and CYC) differed significantly in baseline eGFR levels, with patients in the CYC group having worse renal function at baseline compared to MPA group. In regression analysis and after adjustment for baselineeGFR, neither treatment correlated with time to remission or risk of flare, implying that, in white patients with relatively preserved renal function, both treatments are equally effective.

Several studies have demonstrated that achievement of remission, time till remission and development of flares are factors significantly associated with worse long-term renal outcome (17, 37–41). All patients included in our study achieved remission at some point during the disease course. Interestingly, baseline proteinuria, which has been suggested to predict remission (25), did not seem to affect time to remission in our cohort and neither did the therapeutic regimen applied. Renal function at baseline did not also prove to be a significant predictor of time to remission but it should be noted that 89% (24/27) of the patients had normal renal function (eGFR > 60 ml/min/1.73 m^2) at the time of MLN diagnosis. No other baseline clinical or biochemical parameter has emerged as a significant prognostic factor of time to remission, possibly due to the small number of patients studied.

Renal flares in pure MLN patients range from 22 to 45% in different studies (17, 19, 21, 22) while in our cohort they occurred in only 22% of patients. Proteinuria >1 g/24 h at 1 year after the diagnosis appeared to be the only statistically significant risk factor for flare. This observation adds to the value of 12-month proteinuria, which has, in recent years, emerged as a more reliable predictor of long-term renal outcomes in LN patients (38, 39, 41, 42). Therapeutic regimen (MPA or CYC) did not seem to affect the risk of flare, neither did the time to remission. Lower C3 and C4 levels appeared to increase the risk of flare but not significantly. Larger studies are needed to further examine C3 and C4 as potential determinants of flare occurrence in pure MLN.

There is lack of data in the literature in regard to how the time of onset of MLN affects its clinical presentation as well as its response to treatment and long-term renal outcome. We decided to divide our patients into those presenting with MLN at the time of SLE diagnosis or at some time during the following 12 months ("early" MLN) and into those presenting with MLN afterwards ("late" MLN). Although patients with "early" disease had higher levels of proteinuria at baseline than patients with "late" disease, the former achieved complete remission sooner than the latter, a finding that deserves further investigation.

Our cohort reflects a real-world, uniform management of pure MLN patients followed at a dedicated, specialized center with available data for all patients for a median follow-up period of 77 months. Another strength of the study is its inception cohort design which contributes, par excellence, to the definition of the natural history of a disease and to the determination of correlations between a certain outcome and prognostic factors. Our study has several limitations such as its retrospective nature and mainly, the small number of patients. Such a small sample size is not able to ensure the statistical power of the results, which should be interpreted with caution and evaluated in larger cohorts. Since MLN is a rare entity and progresses rather slowly, multicentric cohorts with longer follow up are needed.

CONCLUSIONS

Pure MLN in our cohort was associated with very good long-term renal outcomes. Mycophenolic acid and cyclophosphamide seemed to be equally effective in means of time to remission and flare prevention. With the limitation of the small sample size, we could not find any baseline clinical or biochemical factor that could predict time to remission. Proteinuria >1 g/24 h at 1 year seemed to be associated with a higher risk of renal flare but this observation should be further examined in larger cohorts.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by Institutional Review Board (IRB) of Laiko General Hospital of Athens and Medical School of National Kapodistrian University of Athens. Written informed consent for participation was not required for this study in accordance with the national legislation and the institutional requirements.

AUTHOR CONTRIBUTIONS

MT, JB, and PS designed the study. EK collected the data and drafted the article. GL reviewed the histological data. IM analyzed the data. SM and MT supervised the process. MT, SM, PS, and JB reviewed the final version of the article. All authors provided contributions and approved the version of the article to be published.

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