KI REPORTS
KIReports.org
CLINICAL RESEARCH

SARS-CoV-2 Protein Deposition Enhances Renal Complement Activation and Aggravates Kidney Injury in Membranous Nephropathy After COVID-19

Guoqin Wang[1], Lei Yang[1], Xiaoyi Xu[1], Weiyi Guo[1], Lijun Sun[1], Yanyan Wang[1], Wenrong Cheng[1], Nan Ye[1], Lingqiang Kong[1], Xiaoyi Zhao[2] and Hong Cheng[1]

[1]Division of Nephrology, Beijing Anzhen Hospital, Capital Medical University, Beijing, China; and [2]Division of Nephrology, Affiliated Hospital of Chifeng University, Neimenggu, China

Introduction: COVID-19 has been reported to be associated with the occurrence and recurrence of membranous nephropathy (MN). The clinicopathological characteristics and complement system activation of MN after COVID-19 are unclear.

Methods: A total of 38 patients with biopsy-proven MN who developed new-onset proteinuria after COVID-19 were enrolled in this study. One hundred patients with primary MN diagnosed before the COVID-19 pandemic were the control. Renal immunohistochemical staining for SARS-CoV-2 nucleocapsid protein was performed in 38 patients with MN after COVID-19. Serum membrane attack complex (MAC) was detected by enzyme-linked immunosorbent assay. Glomerular staining for the complement proteins in different pathways were detected by immunohistochemistry.

Results: Thirteen of 38 patients had positive staining for SARS-CoV-2 nucleocapsid protein. Compared with the control patients, the clinical manifestations were more severe in patients after COVID-19. Patients with positive SARS-CoV-2 staining had a higher proportion of nephrotic syndrome, lower level of serum albumin, and greater severity of renal interstitial fibrosis than those of patients with negative SARS-CoV-2 staining. Serum MAC level and renal MAC staining intensity of MN after COVID-19 were significantly higher than those of the control patients. MAC expression in MN patients with positive SARS-CoV-2 staining was stronger than that in both control patients and MN after COVID-19 with negative SARS-CoV-2 staining. The expression trend of factor H was consistent with that of MAC.

Conclusion: Excessive activation of the complement system aggravated symptoms in MN after COVID-19. Therapeutic strategy targeting the complement system may need to be considered.

Kidney Int Rep (2024) **9,** 3145–3155; https://doi.org/10.1016/j.ekir.2024.08.006
KEYWORDS: complement; coronavirus disease 2019; factor H; membranous nephropathy; severe acute respiratory syndrome corona virus 2; the alternative pathway

After the global COVID-19 pandemic, kidney injury caused by COVID-19 can occur in up to 40% of patients.[1] SARS-CoV-2 infection has been linked to a notable increase in glomerular disease, exacerbation of existing kidney conditions, renal function deterioration, heightened hospitalization rates, increased need for renal replacement therapy, and elevated mortality risk. Notably, numerous cases of glomerular disease have emerged in conjunction with COVID-19, with collapsing glomerulopathy currently being the most common pathology (approximately 36.1%), and MN at approximately 5.6%.[2]

MN is the most common pathological type of adult nephrotic syndrome. It is characterized by the deposition of immune complexes under glomerular epithelial cells, which include specific antigens, IgG, and complement MACs. Majority of MN antigens (70%–80%) are phospholipase A2 receptor (PLA2R), followed by neural EGF-like 1 (NELL-1) and thrombospondin type 1 domain-containing 7A (THSD7A). Approximately 70% of MN are considered primary, whereas the remaining 30% are secondary to various

Correspondence: Hong Cheng, Division of Nephrology, Beijing Anzhen Hospital, Capital Medical University, 2 Anzhen Road, Beijing 100029, China. E-mail: drchengh@163.com
Received 2 February 2024; revised 22 July 2024; accepted 5 August 2024; published online 10 August 2024

etiologies, including infections such as hepatitis virus as one of the important secondary causes.[3] However, the association between COVID-19 and the pathogenesis of MN remains unclear. Studies have suggested that SARS-CoV-2 can induce complement activation and immune dysregulation, leading to kidney injury.[4–7] Furthermore, similar to the detection of hepatitis B antigen in renal tissue of patients with hepatitis B-related MN,[8] it is speculated that SARS-CoV-2 protein deposition in renal tissue might contribute to the formation of immune complex and the subsequent development of MN.[9,10] Although MN associated with COVID-19 has been reported, limited research with detailed patient data is available, especially regarding viral detection and complement activation in renal tissues.[1]

This study collected clinical, prognostic, and pathological data of patients with new-onset MN after COVID-19 in our center and compared with those of patients diagnosed with primary MN before COVID-19 pandemic. We detected SARS-CoV-2 and complement-related indicators in renal biopsy tissue of all patients to explore the role of SARS-CoV-2 protein in complement activation and the worsening of renal injury.

METHODS

Patients

A total of 38 patients with biopsy-proven MN, who developed new-onset proteinuria after COVID-19, were enrolled in this study from December 2022 to July 2023. Secondary MN cases caused by autoimmune diseases (such as systemic lupus erythematosus, rheumatoid arthritis, and Sjogren's syndrome), hepatitis virus infections (hepatitis B and C), and heavy metal poisoning (mercury, etc.) were excluded. The control group consisted of consecutive patients with primary MN confirmed by renal biopsy between December 2018 and July 2019. Baseline clinical data of the patients at admission were collected, and some patients were followed-up with. Serum samples were obtained on the day of kidney biopsy and stored at −80 °C until use. The study was conducted in compliance with the Declaration of Helsinki and approved by the ethics committee of Beijing Anzhen Hospital. Written informed consent was obtained for tissue and blood sampling.

The diagnosis of primary MN was based on immunofluorescence, light microscopy, and electron microscopy. Response to treatment was evaluated according to the Kidney Disease: Improving Global Outcomes guidelines.[11] Complete remission was defined as urinary protein excretion less than 0.3 g/d (urinary albumin-to-creatinine ratio $<$ 300 mg/g or $<$ 30 mg/mmol), confirmed by 2 values at least 1 week apart, accompanied by normal levels of serum albumin and creatinine. Partial remission was defined as urinary protein excretion $<$ 3.5 g/d (urinary albumin-to-creatinine ratio $<$ 3500 mg/g or $<$ 350 mg/mmol), and at least a 50% reduction from peak values, accompanied by improvement or normalization of serum albumin levels and stable serum creatinine levels.

Immunohistochemical Staining of SARS-CoV-2 Nucleocapsid Protein in Renal Tissue

The method used to detect SARS-CoV-2 nucleocapsid protein in renal biopsy was conducted according to previously described procedures.[9,12] In brief, paraffin-embedded biopsy renal tissues were sliced into 4 μm thick sections and then deparaffinized. Antigen retrieval was performed by heating the slides in 1× EDTA buffer (pH 9.0), at 98 °C for 40 minutes. After cooling to room temperature and washing 3 times with phosphate-buffered saline (PBS), the slides were treated with 3% hydrogen peroxide solution for 10 minutes to inhibit endogenous peroxidase activity. Subsequently, the slides were blocked with 3% bovine serum albumin in PBS at room temperature for 30 minutes, followed by incubation with mouse anti-SARS-CoV-2 (2019-nCoV) nucleocapsid antibody (40588-MM137, Sino Biological, China) diluted at 1:200 in PBS overnight at 4 °C. Following 3 washes with PBS for 5 minutes each, antimouse IgG-horseradish peroxidase (KIT-5001, Maixin Biotech, China) was added and incubated for 1 hour at 37 °C. The peroxidase activity was visualized using 3–3′-diaminobenzidine-tetrahydrochloride. Finally, the slides were dehydrated in ethanol and xylene, and sealed using neutral gum.

Immunofluorescence Colocalization Staining of SARS-CoV-2 Nucleocapsid Protein and IgG in Renal Tissues

Frozen tissues of 4 μm thickness were taken and dried in a fuser, then washed 3 times with PBS and added with mouse anti-SARS-CoV-2 (2019-nCoV) nucleocapsid antibody (40588-MM137, Sino Biological, China), with a dilution of 1:200 in PBS, which were incubated overnight at 4 °C. After warming for 30 minutes, TRITC-conjugated goat antimouse IgG (dilution 1:100, CWO152S, Cwbiotech, China) and rabbit polyclonal antihuman IgG/FITC (dilution 1:40, F0202, Dako, Denmark) were added after washing 3 times with PBS, and then incubated at 37 °C for 40 minutes. Finally, the slides were washed 3 times with PBS and imaged under a fluorescence microscope.

Immunofluorescence Stain of H Factor, Factor H-related Protein 5 (FHR-5), MAC, and Mannan-Binding Lectin (MBL) in Renal Tissue

Paraffin-embedded biopsy renal tissues from patients underwent immunofluorescence staining to detect the presence of H factor, FHR-5, MAC, (C5b-9), and MBL, following previously described protocols.[13] The primary antibodies used for the staining included mouse monoclonal anti-Factor H antibody (dilution 1:200, sc-47686, Santa Cruz Biotechnology, CA), rabbit polyclonal anti-FHR-5 antibody (dilution 1:200, GTX109982, GeneTex, Inc., Irvine, CA), mouse monoclonal anti-C5b-9 + C5b-8 antibody (dilution 1:50, ab66768, Abcam, CA), and mouse monoclonal anti-MBL antibody (dilution 1:200, HM2061, Hycult Biotech, Netherlands). The primary antibodies were incubated overnight at a temperature of 4 °C. After being washed, goat antimouse IgG/FITC (ZF-0312, ZSGB-BIO, China) or goat anti-rabbit IgG/FITC (ZF-0311, ZSGB-BIO, China) were added for 1 hour at 37 °C as a secondary antibody. Finally, the sections were washed and air dried in the dark.

The immunofluorescence intensity was independently scored by 2 pathologists. Fluorescence intensity criteria: there is no light at both low and high magnification as "−"; negative at low magnification, seemed to be visible at high magnification as "±"; it seems to be visible at low magnification, and blurred at high magnification as "+"; it is obviously visible at low magnification and clearly visible at high magnification as "++"; clearly visible at low magnification and dazzling fluorescence at high magnification as "+++".[14]

Quantification of Plasma Levels of Soluble MAC

Plasma concentrations of soluble MAC were quantified using enzyme-linked immunosorbent assay kits provided by Quidel Corporation (A020, USA). The measurements were performed following the manufacturer's protocols.

Immunohistochemical Staining of PLA2R, Thrombospondin Type 1 Domain-Containing 7A, and NELL-1 in Renal Tissue

Paraffin-embedded kidney tissue (4 μm) was obtained for analysis. For the retrieval of PLA2R and NELL-1 antigens, a pH 6 citrate solution combined with trypsin was used, whereas a pH 9 EDTA solution was used for thrombospondin type 1 domain-containing 7A. The primary antibodies utilized were rabbit polyclonal antihuman PLA2R1 antibody (HPA012657, Sigma, USA) at a dilution of 1:800, rabbit polyclonal antihuman NELL-1 antibody (HPA051535, Sigma, USA) at a dilution of 1:400, and rabbit polyclonal antihuman thrombospondin type 1 domain-containing 7A antibody (HPA000923, Sigma, USA) at a dilution of 1:3000. These antibodies were incubated overnight at 4 °C. Subsequently, the secondary antibody, either alkaline phosphatase-labeled immunohistochemistry reagent (KIT-5103, Max Vision, China) or horseradish peroxidase-labeled immunohistochemistry reagent (KIT-5004, Max Vision, China), was added and incubated at room temperature for 30 minutes. Fast-Red reagent (Zhongshan Jinqiao, ZLI-9042) or 3–3′-diaminobenzidine-tetrahydrochloride reagent (Zhongshan Jinqiao, ZLI-9018) was utilized for color development.

Statistical Analyses

Statistical analyses were conducted using SPSS 21.0 (SPSS Inc., Chicago, IL). Parametric data were reported as means ± SDs, whereas nonparametric data were presented as median values with 25th to 75th percentiles. Group comparisons for continuous variables were carried out using 1-way analysis of variance or the Mann-Whitney U test (Kruskal-Wallis), and categorical variables were compared using the chi-square test. All P values were 2-tailed, and statistical significance was set at $P < 0.05$.

RESULTS

Clinical and Pathological Characteristics of Patients With MN After COVID-19

A total of 38 patients with new-onset MN after COVID-19 were included in this study (Figure 1). The median duration from the onset of proteinuria to COVID-19 was 1.0 (0.5–3.0) month. Clinical symptoms of COVID-19 among the patients included fever (100%), sore throat (84.2%), cough (31.6%), expectoration (26.3%), and loss of appetite (78.9%). None of the patients had severe pneumonia. At the time of renal biopsy, all patients tested negative for SARS-CoV-2 infection. Of the

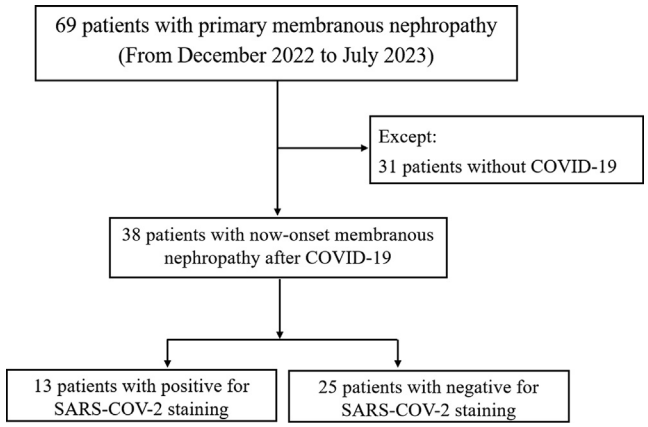

Figure 1. Flow chart of the patients enrolled in the study.

38 patients, 22 (57.9%) were male, with a median age of 55.5 years. In addition, 78.9% of the patients presented with nephrotic syndrome. Compared to 100 patients with primary MN without COVID-19 in the control group, serum albumin level of MN after COVID-19 was significantly lower ($P = 0.011$). Moreover, patients with MN after COVID-19 also displayed an increase in urinary protein and serum creatinine levels, although these differences were not statistically significant ($P = 0.054$ and $P = 0.062$, respectively) (Table 1). There was no significant difference in Ehrenreich-Churg staging and the positivity rate of these antigens between patients with MN after COVID-19 and patients in the control group. The positive rate of PLA2R in renal tissues of MN after COVID-19 was 84.2%, and 3 cases (7.9%) were positive for NELL-1. Notably, compared with patients in the control group, the positivity rate of IgG1 staining (62.2%) and the degree of interstitial fibrosis of MN after COVID-19 was significantly higher, whereas there was no difference in the positivity rate of IgG4 (Table 2). In patients with MN after COVID-19, 18 was followed-up for 6 months; 33.3% of the patients achieved partial remission at 6 months. No patients achieved complete remission. In the control group, 17 of 31 patients followed-up for 6 months achieved partial remission or complete remission. However, due to the short follow-up time, the difference between the 2 groups were not statistically significant (33.3% vs. 54.8%, $P = 0.171$) (Table 3).

Clinical and Pathological Characteristics of Patients Positive for SARS-CoV-2 Staining in Renal Tissues of MN After COVID-19

Immunohistochemistry analysis of SARS-CoV-2 was performed in 38 patients. Thirteen patients exhibited positive staining in the renal tubular epithelial cells (Figure 2i and j). Seven of 13 patients had prominent granular deposition of SARS-CoV-2 nucleocapsid protein along the glomerular basement membrane (Figure 2k and l). Colocalization staining further revealed that the glomerular SARS-CoV-2 staining overlapped with the IgG staining in glomeruli (Figure 3, Supplementary Figure S1 shows images of the negative control). Among the 13 patients, 9 (69.2%) were male. The median time from COVID-19 to the detection of proteinuria was 1.0 (0.3–2.0) month. All patients were presented with nephrotic syndrome. In comparison to patients in the control group, the clinical manifestations were more severe in patients with positive SARS-CoV-2 staining. Serum albumin and estimated glomerular filtration rate levels were lower, whereas urine protein and serum creatinine levels were significantly increased (Table 1). In addition, coagulation indicators were significantly elevated (Table 1). Seven patients positive for SARS-CoV-2 were followed-up for 6 months. However, there was no significant difference in remission rate observed at 6 months compared with the control group (Table 3). Among 13 patients with positive SARS-CoV-2 staining, 2 (15.4%) were in Ehrenreich-Churg stage 1 and 10 (76.9%) in

Table 1. Comparison of clinical characteristics among MN after COVID-19, MN after COVID-19 with positive for SARS-CoV-2 staining, and MN without COVID-19

Characteristics	MN after COVID-19 ($n = 38$)	MN after COVID-19 with positive for SARS-CoV-2 staining (n = 13)	MN without COVID-19 (n = 100)	[a]*P* value	[b]*P* value
Male, *n* (%)	22 (57.9)	9 (69.2)	51 (51.0)	0.469	0.251
Age (yr)	55.5 (41.0–64.5)	56.0 (45.0–64.5)	54.5 (39.0–61.8)	0.233	0.315
Hypertension, *n* (%)	17 (44.7)	5 (38.5)	40 (40.0)	0.614	1.000
Microscopic hematuria, *n* (%)	21 (61.8)	7 (63.6)	43 (43.0)	0.058	0.217
Proteinuria (g/24 h)	5.8 (3.6,9.0)	7.3 (4.8,10.2)	4.3 (2.7,6.8)	0.054	0.006
Serum albumin, (g/l)	25.7 (20.4–30.0)	24.8 (18.0–26.1)	28.1 (23.2–33.1)	0.011	0.002
Nephrotic syndrome, *n* (%)	30 (78.9)	13 (100.0)	64 (64.0)	0.092	0.009
Serum creatinine (μmol/l)	69.0 (57.0–92.0)	84.0 (62.5–109.5)	63.9 (54.2–76.4)	0.062	0.005
eGFR-EPI (ml/min per 1.73 m^2)	97.6 (79.6–109.8)	82.7 (61.8–98.3)	100.6 (93.2–111.5)	0.079	0.004
Triglycerides (mmol/l)	2.1 (1.4–3.5)	2.0 (1.7–5.2)	2.1 (1.4–3.2)	0.874	0.437
Total cholesterol (mmol/l)	7.7 (6.1–8.6)	8.2 (6.2–10.1)	6.7 (5.6–8.6)	0.204	0.192
LDL (mmol/l)	4.9 (3.8–6.2)	5.3 (3.3–6.6)	4.0 (2.8–5.4)	0.020	0.240
Uric acid (μmol/l)	320.7 (277.4–370.0)	351.0 (281.0–438.2)	348.0 (285.0–427.0)	0.282	0.864
D-D (ng/ml)	781.0 (238.8–1557.5)	1230.0 (781.0–2600.0)	400.0 (185.5–900.0)	0.145	0.003
FDP (μg/ml)	4.2 (2.1–6.9)	6.4 (3.4–8.4)	2.0 (1.0–4.1)	0.005	0.007
serum PLA2R, *n* (%)	22 (57.9)	8 (61.5)	53 (53.0)	0.470	0.288
serum PLA2R (RU/ml)	51.4 (3.8–278.0)	82.2 (3.3–301.5)	26.6 (0.6–95.5)	0.156	0.121

D-D, D-dimer; eGFR-EPI, estimated glomerular filtration rate using the Epidemiology Collaboration formula; FDP, fibrin/fibrinogen degradation products; IQR, interquartile range; LDL, low density lipoprotein cholesterol; MN, membranous nephropathy.
[a]P value: MN after COVID-19 vs. MN without COVID-19.
[b]P value: MN after COVID-19 with positive for SARS-CoV-2 staining vs. MN without COVID-19.
Data are given as *n* (%) or median (IQR) unless otherwise noted.

Table 2. Comparison of pathological characteristics among MN after COVID-19, MN after COVID-19 with positive for SARS-CoV-2 staining, and MN without COVID-19

Characteristics	MN after COVID-19 (n = 38)	MN after COVID-19 with positive for SARS-CoV-2 staining (n = 13)	MN without COVID-19 (n = 100)	[a]*P* value	[b]*P* value
Ehrenreich-Churg stage				0.061	0.105
Stage 1, *n* (%)	9 (23.7)	2 (15.4)	42 (42.0)		
Stage 2, *n* (%)	26 (68.4)	10 (76.9)	46 (46.0)		
Stage 3, *n* (%)	3 (7.9)	1 (7.7)	12 (12.0)		
MN antigen				0.852	0.775
PLA2R positive, *n* (%)	32 (84.2)	10 (76.9)	86 (86.0)		
NELL-1 positive, *n* (%)	3 (7.9)	1 (7.7)	5 (5.0)		
THSD7A positive, *n* (%)	0	0	1 (1.0)		
IgG positive, *n* (%)	38 (100.0)	13 (100.0)	99 (99.0)	1.000	1.000
IgA positive, *n* (%)	2 (5.3)	0	11 (11.0)	0.481	0.358
IgM positive, *n* (%)	2 (5.3)	0	11 (11.0)	0.481	0.358
IgG1 positive, *n* (%)	23 (62.2)	7 (53.8)	39 (39.0)	0.016	0.373
IgG2 positive, *n* (%)	1 (2.7)	0	7 (7.0)	0.588	1.000
IgG3 positive, *n* (%)	1 (2.7)	0	11 (11.0)	0.236	0.358
IgG4 positive, *n* (%)	31 (83.8)	11 (84.6)	92 (92.0)	0.275	0.323
TA/IF, median (IQR)	1.0 (1.0,2.0)	2.0 (1.0,2.0)	1.0 (1.0,1.0)	<0.001	<0.001
FSGS, *n* (%)	3 (7.9)	1 (7.7)	5 (5.0)	0.809	0.528

FSGS, focal segmental glomerulosclerosis; IF, interstitial fibrosis; IQR, interquartile range; MN, membranous nephropathy; NELL-1, neural EGF-like 1; PLA2R, phospholipase A2 receptor; TA, tubular atrophy; THSD7A, thrombospondin type 1 domain-containing 7A.
[a]*P* value: MN after COVID-19 vs. MN without COVID-19.
[b]*P* value: MN after COVID-19 with positive for SARS-CoV-2 staining vs. MN without COVID-19.
Immunofluorescence values 2+ and above are positive.

stage 2, with only 1 patient (7.7%) at stage 3. Ten patients (76.9%) were positive for PLA2R, and 1 patient (7.7%) was positive for NELL-1. The degree of renal interstitial fibrosis in renal MN positive for SARS-CoV-2 staining was significantly higher than that of patients in the control group (Table 2).

The characteristics of 38 patients with MN after COVID-19 with or without positive SARS-CoV-2 staining were compared. The results show that patients with positive SARS-CoV-2 staining had more severe baseline clinical manifestations, a higher proportion of nephrotic syndrome, and lower level of serum albumin (Supplementary Table S1). The severity of renal interstitial fibrosis was also significantly greater (Supplementary Table S2). No difference in the remission rate was found at 6 months after treatment (Supplementary Table S3). The clinical and pathological characteristics between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell were also compared. No statistically significant differences were observed between the 2 groups (Supplementary Table S4–6).

Changes of Complement-Related Indicators in Serum of Patients with MN After COVID-19

Compared with patients without COVID-19 in the control group, serum complement C3 and C4 levels of MN after COVID-19 were significantly higher. Similarly, serum complement C3 level of patients with positive for SARS-CoV-2 staining were significantly higher than that of control group. In addition, the levels of soluble MAC were examined by enzyme-linked immunosorbent assay. There was a marked increase in both 38 patients with MN after COVID-19 and 13 patients with MN positive for SARS-CoV-2 staining

Table 3. Comparison of treatment and prognosis among MN after COVID-19, MN after COVID-19 with positive for SARS-CoV-2 staining, and MN without COVID-19

Characteristics	MN after COVID-19 (n = 18)	MN after COVID-19 with positive for SARS-CoV-2 staining (n = 7)	MN without COVID-19 (n = 31)	[a]*P* value	[b]*P* value
Immunosuppressive therapy, *n* (%)	15 (83.3)	7 (100.0)	25 (80.6)	1.000	0.305
Prognosis				0.171	0.583
Partial remission, *n* (%)	6 (33.3)	3 (42.9)	13 (41.9)		
Complete remission, *n* (%)	0	0	4 (12.9)		

MN, membranous nephropathy.
[a]*P* value: MN after COVID-19 vs. MN without COVID-19.
[b]*P* value: MN after COVID-19 with positive for SARS-CoV-2 staining vs. MN without COVID-19.

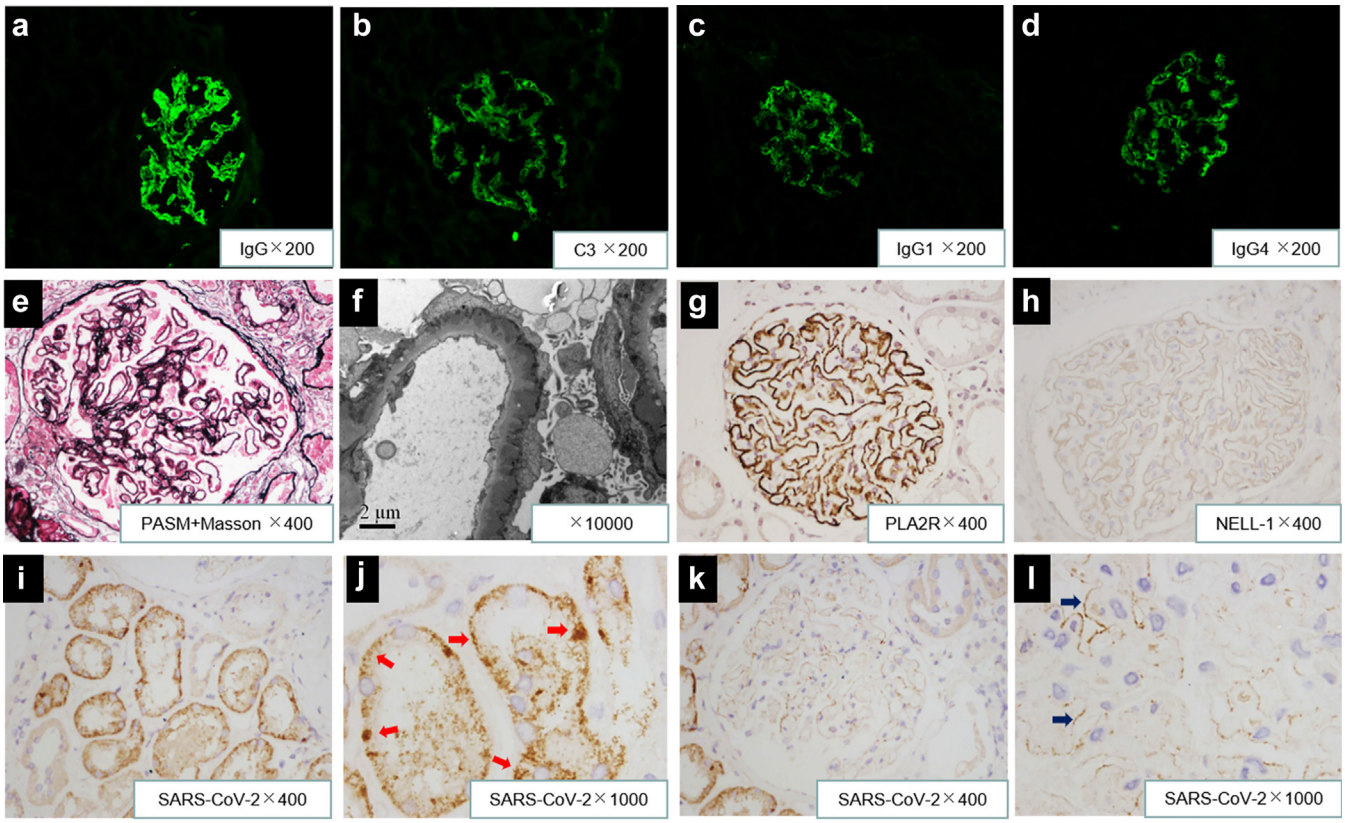

Figure 2. The renal pathologic images of a patient with PLA2R-positive MN patient after COVID-19 who also is positive for SARS-CoV-2 staining. Immunofluorescence staining demonstrated positive for (a) IgG and (b) complement C3 along the glomerular basement membrane in the renal biopsy specimen from a patient with MN after COVID-19 (×200). IgG subclass staining revealed positive for both (c) IgG1 and (d) IgG4 (×200). (e) Subepithelial eosinophil deposits on PASM-Masson staining were observed under light microscopy (×400). (f) Electron microscopy showed subepithelial electron dense deposits (×10000). (g) Glomerular PLA2R staining was positive, whereas (h) NELL-1 staining was negative (×400). Immunohistochemical staining for SARS-CoV-2 nucleocapsid antibody (dilution 1:200) was expressed (i, j) in the renal tubular epithelial cells (red arrows) and (k, l) along the glomerular basement membrane (blue arrows) (×400, ×1000).

compared with patients in the control group (Table 4). These results indicate a notable activation of the complement system in the serum of patients with MN after COVID-19.

Serum levels of complement C3, C4, and soluble MAC were compared between patients with positive and negative for SARS-CoV-2 staining and there were no significant differences (Supplementary Table S7).

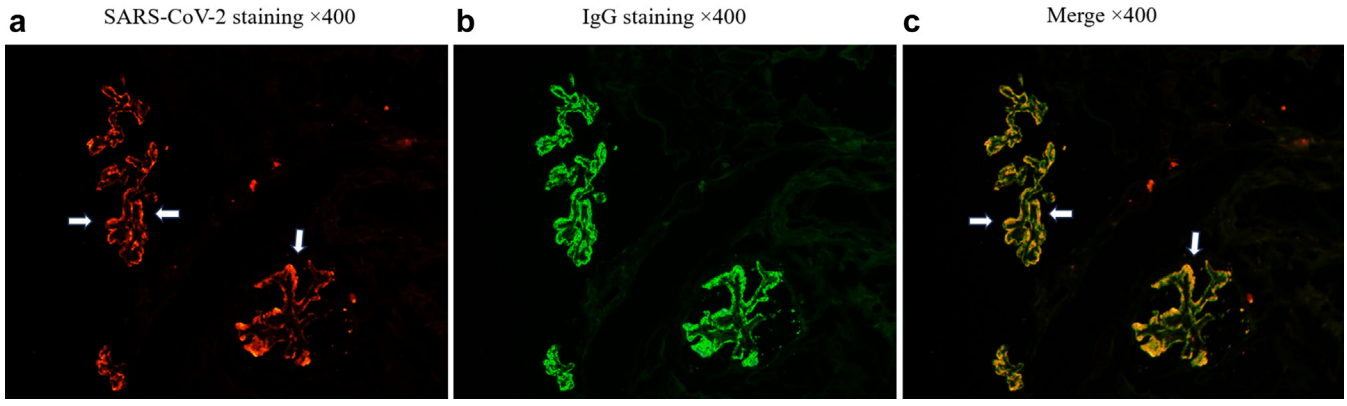

Figure 3. Colocalization of SARS-CoV-2 nucleocapsid staining and IgG staining in glomeruli in a patient with positive for SARS-CoV-2 staining. Frozen renal tissue was incubated with mouse anti-SARS-CoV-2 nucleocapsid antibody (1:200), then TRITC conjugated goat antimouse IgG (dilution) and rabbit polyclonal anti-human IgG/FITC (dilution) were added as second antibody. (a, white arrows) The expression of SARS-CoV-2 nucleocapsid protein shows segmental positivity along the glomerular basement membrane and is in red (b) The expression of IgG is shown in green (c) Coexpression of SARS-CoV-2 nucleocapsid protein and IgG is shown along the glomerular basement membrane by the merge of the fluorescence in yellow.

Table 4. Comparison of complement-related indicators in serum and in renal tissue among MN after COVID-19, MN after COVID-19 with positive for SARS-CoV-2 staining, and MN without COVID-19

Characteristics	MN after COVID-19 (n = 38)	MN after COVID-19 with positive for SARS-CoV-2 staining (n = 13)	MN without COVID-19 (n = 100)	[a]*P* value	[b]*P* value
Serum complement C3, median (IQR)	1.4 (1.1–1.5)	1.4 (1.2–1.7)	1.2 (1.0–1.4)	0.007	0.006
Serum complement C4, median (IQR)	0.31 (0.3–0.4)	0.33 (0.2–0.4)	0.27 (0.2–0.3)	0.015	0.085
sMAC (ng/ml), median (IQR)	1646.6 (1333.5–2100.0)	1810.4 (1398.2–2047.3)	1195.8 (859.7–1514.0)	0.001	0.002
C3 positive, *n* (%)	23 (60.5)	6 (46.2)	67 (67.0)	0.476	0.216
C1q positive, *n* (%)	1 (2.6)	1 (7.7)	5 (5.0)	0.887	0.528

IQR, interquartile range; MN, membranous nephropathy; sMAC, soluble membrane attack complex.
[a]*P* value: MN after COVID-19 versus MN without COVID-19.
[b]*P* value: MN after COVID-19 with positive for SARS-CoV-2 staining versus MN without COVID-19.
Immunofluorescence values 2+ and above are positive.

Similarly, there was no statistically significant difference between MN with positive SARS-CoV-2 staining in tubular cells and those with positive SARS-CoV-2 staining in both glomeruli and tubular cells (Supplementary Table S8).

Changes of Complement-Related Indicators in Renal Tissues of Patients With MN After COVID-19

Immunofluorescence staining showed no significant difference in the positive rate of renal C3 staining among the various groups. Therefore, immunofluorescence staining was performed to detect MAC in glomeruli (Supplementary Figure S2). The results showed that the intensity of MAC in MN after COVID-19 was significantly higher compared to that of patients in the control group (Figure 4). In addition, patients with MN who were positive for SARS-CoV-2 staining exhibited significantly stronger than that of patients in either the control group or MN after COVID-19 negative for SARS-CoV-2 staining (Figure 4). These findings suggest that the complement activation

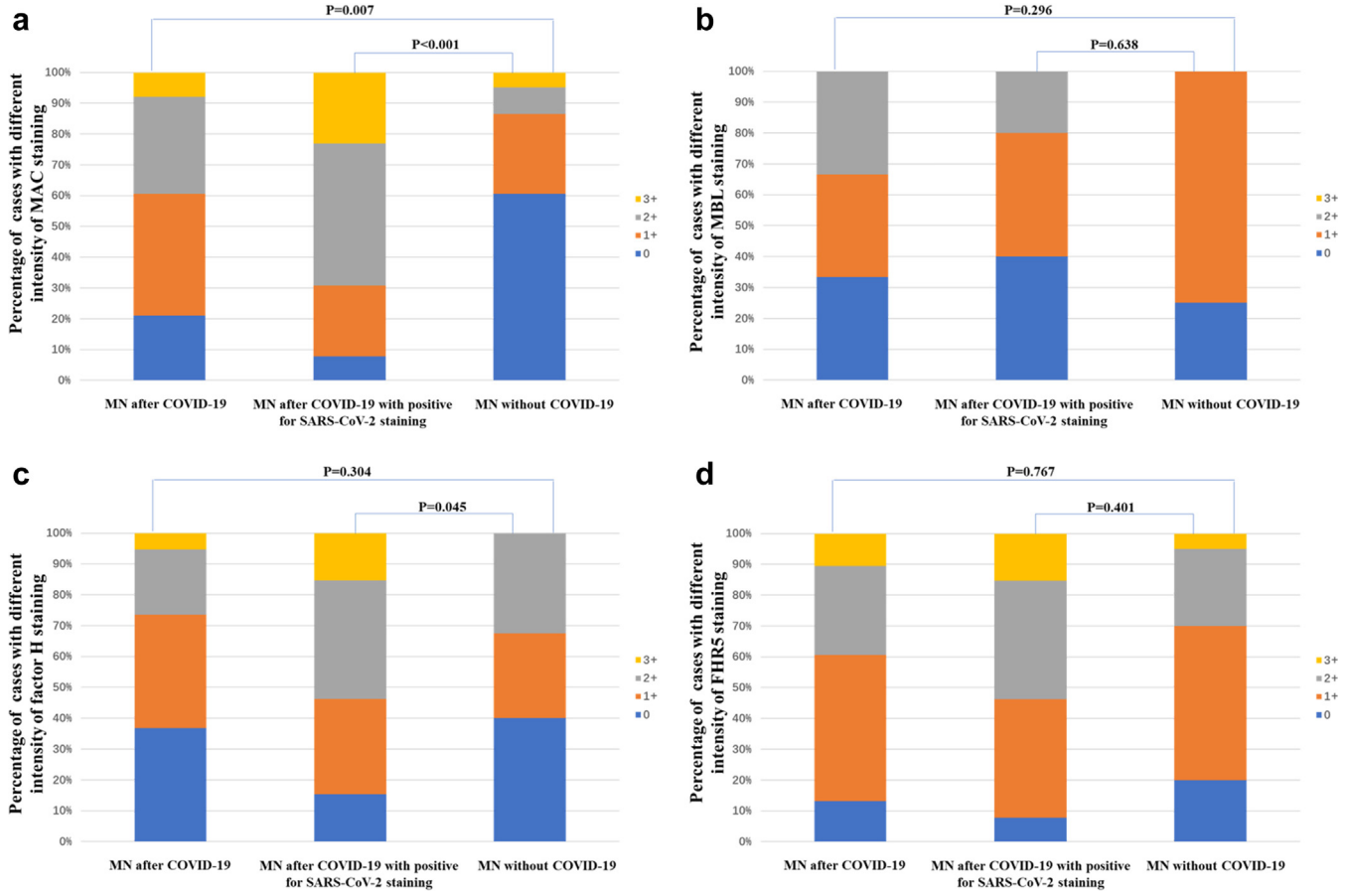

Figure 4. Comparison of the staining intensity of MAC, MBL, factor H, and FHR-5 in renal tissues. Bar graph shows the comparison of percentage of case with different intensity in (a) MAC, (b) MBL, (c) factor H, and (d) FHR-5. FHR-5, factor H-related protein 5; MAC, membrane attack complex; MBL, mannan-binding lectin.

in the renal tissue of MN after COVID-19 was stronger compared with that in MN without COVID-19 and leading to the elevation of the MAC. Furthermore, the presence of SARS-CoV-2 protein in renal tissue may further enhance complement activation and contribute to the exacerbation of kidney injury (Figure 4).

The complement proteins in different pathways were detected by immunofluorescence staining (Supplementary Figure S2). The staining intensity of C1q in the classical pathway, MBL in the lectin pathway, factor H, and FHR-5 in the alternative pathway showed no significant difference between MN after COVID-19 and patients in the control group. However, compared to patients in the control group, the intensity of factor H in renal tissue of MN after COVID-19 with positive for SARS-CoV-2 staining was significantly higher, and no significant difference in the intensity of FHR-5, C1q, and MBL (Table 4 and Figure 4). Notably, the intensity of factor H was significantly greater in 13 patients with positive for SARS-CoV-2 staining than 25 patients with negative for SARS-CoV-2 staining (Supplementary Figure S3). It suggested that the complement alternative pathway activation may be present in renal tissue of patients with positive for SARS-CoV-2 staining. The complement-related indicators in renal tissue between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell were compared and there were not significantly differences (Supplementary Table S9).

DISCUSSION

It has been reported that various glomerular diseases can arise after COVID-19. Early studies of COVID-19 did not find the evidence of SARS-CoV-2 in kidney tissue.[2,15-17] Nevertheless, subsequent investigations have identified the presence of SARS-CoV-2 and angiotensin-converting enzyme 2 in urine, as well as SARS-CoV-2 in kidney tissue, providing support for the kidney as one of the target organs affected by SARS-CoV-2.[9,18,19] Therefore, in this study, we conducted SARS-CoV-2 staining on renal biopsy tissues of 38 patients who experienced the onset of MN after COVID-19. Out of these patients, 13 were positive for SARS-CoV-2 in renal tissues. This finding suggests a need for further investigation into the relationship between MN and SARS-CoV-2 infection.

In previous studies, SARS-CoV-2 has been found primarily in renal tubular epithelial cells of patients with COVID-19 with kidney injury.[9,20-22] In this study, we observed deposits of SARS-CoV-2 nucleocapsid protein along the glomerular basement membrane for the first time. Furthermore, immunofluorescence confocal microscopy confirmed colocalization of SARS-CoV-2 nucleocapsid protein and IgG in the immune deposits. The presence of SARS-CoV-2 in glomerular podocytes was also confirmed through electron microscopy in previous study reports by Su *et al*.,[9] as well as through *in situ* hybridization by Amann *et al*.[23] These findings provide evidence that SARS-CoV-2 is capable of directly invading renal podocytes. In addition, key structural components involved in the SARS-CoV-2 infection of host cells, angiotensin-converting-enzyme 2 and angiotensin-converting-enzyme transmembrane protease serine 2, have been found to be expressed in podocytes.[24,25] Therefore, our study suggests that SARS-CoV-2 infection may contribute to the deposition of viral protein under epithelial cells and lead to podocyte injury. However, the relationship between the SARS-CoV-2 protein deposition, and the pathogenesis of MN remains unclear and needs further investigation in future foundational research.[10]

There have been few reports about cases of MN after COVID-19. Patients typically presented with nephrotic range proteinuria, approximately 80% accompanied by hematuria, and about 50% accompanied by renal insufficiency or acute kidney injury.[1,10,26-29] Our study reported the largest number of cases of MN after COVID-19. Consistent with previous studies, 78.9% of 38 patients with MN after COVID-19 were with nephrotic syndrome, and over 60% had hematuria. However, the proportion of patients with renal insufficiency and acute kidney injury in our study was low, likely due to the mild systemic symptoms of COVID-19 in these patients. The median time from COVID-19 to nephropathy diagnosis was 1.0 month, with an interval exceeding 2 months in a small number of patients. Notably, for the first time, we compared the clinical data of MN after COVID-19 with primary MN without COVID-19, revealing that the clinical manifestations of MN after COVID-19 were more severe. Specifically, urinary protein, serum albumin, and serum creatinine levels in patients with MN with positive SARS-CoV-2 staining differed significantly from patients with MN without COVID-19, as did serum albumin levels and the proportion of nephrotic syndrome when compared to patients with MN after COVID-19 with negative SARS-CoV-2 staining. These findings suggest that the deposition of SARS-CoV2 protein in tissues can further aggravate the condition. In our study, we compared the clinical and pathological characteristics between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell. No statistically significant differences were observed. This may be attributed to the limited number of patients. In addition, SARS-CoV-2

imposes kidney injury via several mechanisms, including direct viral invasion, endothelial cell damage, immune dysregulation, complement activation, coagulopathy, and renin-angiotensin-aldosterone system dysfunction.[1] A larger sequential study is required to further establish the impact of the deposition sites of SARS-CoV-2 protein on kidney injury.

It is well-established that COVID-19 can trigger activation of the systemic complement system.[30] Patients with COVID-19, particularly those with severe clinical manifestations, exhibit significantly elevated levels of serum C3a, C4d, C3bc, C5a, and MAC.[31-33] MAC is the terminal product of the 3 complement pathways and demonstrates a significant correlation with respiratory failure and systemic inflammation.[34,35] In our study, we observed that MN after COVID-19 had significantly higher levels of serum complement C3, C4, and MAC compared to patients with MN without COVID-19, especially those with positive staining for SARS-CoV-2. The activation of serum complement may explain why the kidney injury in patients with MN after COVID-19 is more severe than that in patients with MN without COVID-19. Furthermore, complement activation induced by immune complex deposition in renal tissues plays an important role in MN pathogenesis. However, the precise initial pathway of complement activation remains unclear. The components from all 3 complement pathways (classical, lectin, and alternative pathways) were found in renal biopsies from patients with MN, and the role of these pathways in MN pathogenesis has been reported.[36-38] Various studies have confirmed that high expression of MAC in podocytes was an essential marker of podocyte injury and aggravation of nephropathy.[38,39] In our study, we assessed MAC expression in renal tissues. The results revealed significantly stronger MAC expression in patients with MN after COVID-19 compared to patients with MN without COVID-19. Exceptionally, MAC expression in patients with MN with positive staining for SARS-CoV-2 was stronger than both that in patients with MN without COVID-19 and those with MN after COVID-19 with negative staining for SARS-CoV-2. Meanwhile, the expression of components from all 3 complement pathways were detected in renal tissues. The expression trend of factor H was consistent with that of MAC, whereas those in C1q and MBL were not. Because factor H is a key regulatory factor in the alternative pathway but not a component, and the sample size in this study is very small. Future studies are necessary to confirm whether the alternative complement pathway is involved in the enhanced deposition of the MAC due to the presence of SARS-CoV-2 protein in renal tissues, consequently leading to more severe kidney injury. In addition, although IgG4 positivity was predominant in patients with MN with COVID-19, the positive rate of glomerular IgG1 was up to 62.2%. The subclasses of IgG display distinct complement activation profiles. Further basic research is required to confirm the role of abnormal IgG subclass distribution.

Limitations exist for this study. The number of MN after COVID-19, especially patients with positive staining for SARS-CoV-2, was small, and this may lead to statistical bias. In addition, the detection of SARS-CoV-2 in renal tissue was solely performed using immunohistochemistry and immunofluorescence, which could potentially result in the exclusion of a few patients.

This study demonstrated that SARS-CoV-2 protein was not only expressed in renal tubular epithelial cells but also deposited along the glomerular basement membrane. SARS-CoV-2 protein was found to colocalize with IgG in the glomeruli, suggesting that viral infection on podocytes may contribute to the subepithelial deposition of viral antigens, leading to podocyte damage. Notably, the clinical manifestations of MN after COVID-19, particularly those with positive staining for SARS-CoV-2 appeared to be more severe compared to patients without COVID-19. The presence of SARS-CoV-2 protein in renal tissue increased the expression of MAC in podocytes, potentially explaining aggravation of kidney injury. Therefore, for MN associated with COVID-19, careful monitoring and follow-up observation are warranted. According to the clinical and pathological features, a treatment strategy targeting the complement system may need to be considered.

DISCLOSURE

All the authors declared no competing interests.

ACKNOWLEDGMENTS

This work was supported by grants from the Capital's Funds for Health Improvement and Research (2022-2-2066). We acknowledge the division of nephrology in Qinghai University-Affiliated Hospital for their contributions to this study.

SUPPLEMENTARY MATERIAL

Supplementary File (PDF)

Figure S1. Image of negative control for SARS-CoV-2 nucleocapsid protein immunofluorescence and immunohistochemistry staining.

Figure S2. Immunofluorescence staining images of MAC, MBL, factor H, and CRF-5 in glomeruli.

Figure S3. Comparison of the staining intensity of MAC, MBL, factor H, and FHR-5 in renal tissues between MN

after COVID-19 with and without positive for SARS-CoV-2 staining.

Table S1. Comparison of clinical characteristics between MN after COVID-19 with and without positive for SARS-CoV-2 staining.

Table S2. Comparison of pathological characteristics between MN after COVID-19 with and without positive for SARS-CoV-2 staining.

Table S3. Comparison of treatment and prognosis between MN after COVID-19 with and without positive for SARS-CoV-2 staining.

Table S4. Comparison of clinical characteristics between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell.

Table S5. Comparison of pathological characteristics between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell.

Table S6. Comparison of treatment and prognosis between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell.

Table S7. Comparison of complement-related indicators in serum and in renal tissue between MN after COVID-19 with and without positive for SARS-CoV-2 staining

Table S8. Comparison of complement-related indicators in serum and in renal tissue between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell.

Table S9. Comparison of complement-related indicators in renal tissue between MN with positive for SARS-CoV-2 staining in tubular cell and those with positive for SARS-CoV-2 staining in glomeruli and tubular cell.

REFERENCES

1. Klomjit N, Zand L, Cornell LD, Alexander MP. COVID-19 and glomerular diseases. *Kidney Int Rep*. 2023;8:1137–1150. https://doi.org/10.1016/j.ekir.2023.03.016
2. Turner-Stokes T, Edwards H, Lightstone L. COVID-19 in patients with glomerular disease. *Curr Opin Nephrol Hypertens*. 2022;31:191–198. https://doi.org/10.1097/MNH.0000000000000769
3. Sethi S, Beck LH Jr, Glassock RJ, et al. Mayo Clinic consensus report on membranous nephropathy: proposal for a novel classification. *Kidney Int*. 2023;104:1092–1102. https://doi.org/10.1016/j.kint.2023.06.032
4. Hilton J, Boyer N, Nadim MK, Forni LG, Kellum JA. COVID19 and acute kidney injury. *Crit Care Clin*. 2022;38:473–489. https://doi.org/10.1016/j.ccc.2022.01.002
5. Han X, Ye Q. Kidney involvement in COVID-19 and its treatments. *J Med Virol*. 2021;93:1387–1395. https://doi.org/10.1002/jmv.26653
6. Afzali B, Noris M, Lambrecht BN, Kemper C. The state of complement in COVID-19. *Nat Rev Immunol*. 2022;22:77–84. https://doi.org/10.1038/s41577-021-00665-1
7. Ali YM, Ferrari M, Lynch NJ, et al. Lectin pathway mediates complement activation by SARS-CoV-2 proteins. *Front Immunol*. 2021;12:714511. https://doi.org/10.3389/fimmu.2021.714511
8. Wang R, Wu Y, Zheng B, et al. Clinicopathological characteristics and prognosis of hepatitis B associated membranous nephropathy and idiopathic membranous nephropathy complicated with hepatitis B virus infection. *Sci Rep*. 2021;11:18407. https://doi.org/10.1038/s41598-021-98010-y
9. Su H, Yang M, Wan C, et al. Renal histopathological analysis of 26 postmortem findings of patients with COVID-19 in China. *Kidney Int*. 2020;98:219–227. https://doi.org/10.1016/j.kint.2020.04.003
10. Ma Q, Li X, Xu G. New-Onset and Relapsed Membranous Nephropathy post SARS-CoV-2 and COVID-19 Vaccination. *Viruses*. 2022;14:2143. https://doi.org/10.3390/v14102143
11. Kidney Disease: Improving Global Outcomes (KDIGO) Glomerulonephritis Work Group. KDIGO clinical practice guideline for glomerulonephritis. *Kidney Int Suppl*. 2012;2:139–274.
12. Schurink B, Roos E, Radonic T, et al. Viral presence and immunopathology in patients with lethal COVID-19: a prospective autopsy cohort study. *Lancet Microbe*. 2020;1:e290–e299. https://doi.org/10.1016/S2666-5247(20)30144-0
13. Guo WY, Sun LJ, Dong HR, et al. Glomerular complement factor H-related protein 5 is associated with histologic injury in immunoglobulin A nephropathy. *Kidney Int Rep*. 2021;6:404–413. https://doi.org/10.1016/j.ekir.2020.11.019
14. Yang L, Wang G, Ye N, et al. Clinicopathological and prognostic characteristics of idiopathic membranous nephropathy with dual antigen positivity. *Front Immunol*. 2024;14:1297107. https://doi.org/10.3389/fimmu.2023.1297107
15. May RM, Cassol C, Hannoudi A, et al. A multi-center retrospective cohort study defines the spectrum of kidney pathology in Coronavirus 2019 Disease (COVID-19). *Kidney Int*. 2021;100(6):1303–1315. https://doi.org/10.1016/j.kint.2021.07.015
16. Shetty AA, Tawhari I, Safar-Boueri L, et al. COVID-19- associated glomerular disease. *J Am Soc Nephrol*. 2021;32:33–40. https://doi.org/10.1681/ASN.2020060804
17. Hatmal MM, Alshaer W, Al-Hatamleh MAI, et al. Comprehensive structural and molecular comparison of spike proteins of SARS-CoV-2, SARS-CoV and MERS-CoV, and their interactions with ACE2. *Cells*. 2020;9:2638. https://doi.org/10.3390/cells9122638
18. Hamming I, Timens W, Bulthuis ML, Lely AT, Navis G, van Goor H. Tissue distribution of ACE2 protein, the functional receptor for SARS coronavirus. A first step in understanding SARS pathogenesis. *J Pathol*. 2004;203:631–637. https://doi.org/10.1002/path.1570
19. Chavan S, Mangalaparthi KK, Singh S, et al. Mass Spectrometric Analysis of urine from COVID-19 Patients for Detection of SARS-CoV-2 viral antigen and to study host response. *J Proteome Res*. 2021;20:3404–3413. https://doi.org/10.1021/acs.jproteome.1c00391
20. Diao B, Wang C, Wang R, et al. Human kidney is a target for novel severe acute respiratory syndrome coronavirus 2 infection. *Nat Commun*. 2021;12:2506. https://doi.org/10.1038/s41467-021-22781-1

21. Bouquegneau A, Erpicum P, Grosch S, et al. COVID-19-associated nephropathy includes tubular necrosis and capillary congestion, with evidence of SARS-CoV-2 in the nephron. *Kidney360*. 2021;2:639–652. https://doi.org/10.34067/KID.0006992020
22. Hassler L, Reyes F, Sparks MA, Welling P, Batlle D. Evidence for and against direct kidney infection by SARS-COV-2 in patients with COVID-19. *Clin J Am Soc Nephrol*. 2021;16:1755–1765. https://doi.org/10.2215/CJN.04560421
23. Amann K, Boor P, Wiech T, et al. COVID-19 effects on the kidney. *Pathologe*. 2021;42:76–80. https://doi.org/10.1007/s00292-020-00900-x
24. Lely LAT, Hamming I, van Goor H, Navis GJ. Renal ACE2 expression in human kidney disease. *J Pathol*. 2004;204:587–593. https://doi.org/10.1002/path.1670
25. May RM, Cassol C, Hannoudi A, et al. A multi-center retrospective cohort study defines the spectrum of kidney pathology in coronavirus 2019 Disease (COVID-19). *Kidney Int*. 2021;100:1303–1315. https://doi.org/10.1016/j.kint.2021.07.015
26. Kudose S, Batal I, Santoriello D, et al. Kidney biopsy findings in patients with COVID-19. *J Am Soc Nephrol*. 2020;31:1959–1968. https://doi.org/10.1681/ASN.2020060802
27. Ferlicot S, Jamme M, Gaillard F, et al. The spectrum of kidney biopsies in hospitalized patients with COVID-19, acute kidney injury, and/or proteinuria. *Nephrol Dial Transplant*. 2021;36:1253–1262. https://doi.org/10.1093/ndt/gfab042
28. Mateus C, Theias Manso R, Martins AR, Branco PQ. Membranous nephropathy after a recent SARS-CoV-2 infection. *BMJ Case Rep*. 2023;16:e252468. https://doi.org/10.1136/bcr-2022-252468
29. Nasr SH, Alexander MP, Cornell LD, et al. Kidney biopsy findings in patients with COVID-19, kidney injury, and proteinuria. *Am J Kidney Dis*. 2021;77:465-446. https://doi.org/10.1053/j.ajkd.2020.11.002
30. Pires BG, Calado RT. Hyper-inflammation and complement in COVID-19. *Am J Hematol*. 2023;98:S74–S81. https://doi.org/10.1002/ajh.26746
31. Yu J, Gerber GF, Chen H, et al. Complement dysregulation is associated with severe COVID-19 illness. *Haematologica*. 2022;107:1095–1105. https://doi.org/10.3324/haematol.2021.279155
32. Yu J, Yuan X, Chen H, Chaturvedi S, Braunstein EM, Brodsky RA. Direct activation of the alternative complement pathway by SARS-CoV-2 spike proteins is blocked by factor D inhibition. *Blood*. 2020;136:2080–2089. https://doi.org/10.1182/blood.2020008248
33. Satyam A, Tsokos MG, Brook OR, Hecht JL, Moulton VR, Tsokos GC. Activation of classical and alternative complement pathways in the pathogenesis of lung injury in COVID-19. *Clin Immunol*. 2021;226:108716. https://doi.org/10.1016/j.clim.2021.108716
34. de Nooijer AH, Grondman I, Janssen NAF, et al. Complement activation in the disease course of COVID-19 and its effects on clinical outcomes. *J Infect Dis*. 2021;223:214–224. https://doi.org/10.1093/infdis/jiaa646
35. Holter JC, Pischke SE, de Boer E, et al. Systemic complement activation is associated with respiratory failure in COVID-19 hospitalized patients. *Proc Natl Acad Sci U S A*. 2020;117:25018–25025. https://doi.org/10.1073/pnas.2010540117
36. Seifert L, Zahner G, Meyer-Schwesinger C, et al. The classical pathway triggers pathogenic complement activation in membranous nephropathy. *Nat Commun*. 2023;14:473. https://doi.org/10.1038/s41467-023-36068-0
37. Łukawska E, Polcyn-Adamczak M, Niemir ZI. The role of the alternative pathway of complement activation in glomerular diseases. *Clin Exp Med*. 2018;18:297–318. https://doi.org/10.1007/s10238-018-0491-8
38. Brglez V, Boyer-Suavet S, Seitz-Polski B. Complement pathways in membranous nephropathy: complex and multifactorial. *Kidney Int Rep*. 2020;5:572–574. https://doi.org/10.1016/j.ekir.2020.02.1033
39. So BYF, Chan GCW, Yap DYH, Chan TM. The role of the complement system in primary membranous nephropathy: a narrative review in the era of new therapeutic targets. *Front Immunol*. 2022;13:1009864. https://doi.org/10.3389/fimmu.2022.1009864