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Complement activation and increased expression of Syk, mucin-1 and CaMK4 in kidneys of patients with COVID-19

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ABSTRACT

Acute and chronic kidney failure is common in hospitalized patients with COVID-19, yet the mechanism of injury and predisposing factors remain poorly understood. We investigated the role of complement activation by determining the levels of deposited complement components (C1q, C3, FH, C5b-9) and immunoglobulin along with the expression levels of the injury-associated molecules spleen tyrosine kinase (Syk), mucin-1 (MUC1) and calcium/calmodulin-dependent protein kinase IV (CaMK4) in the kidney tissues of people who succumbed to COVID-19. We report increased deposition of C1q, C3, C5b-9, total immunoglobulin, and high expression levels of Syk, MUC1 and CaMK4 in the kidneys of COVID-19 patients. Our study provides strong rationale for the expansion of trials involving the use of inhibitors of these molecules, in particular C1q, C3, Syk, MUC1 and CaMK4 to treat patients with COVID-19.

1. Introduction

Coronavirus disease 2019 (COVID-19), a pandemic caused by coronavirus 2 (SARS-CoV-2) has affected the globe with devastating societal and economic consequences [1,2]. Severe COVID-19 symptoms include respiratory distress, venous thrombosis, and failure of vital organs [3–7]. In particular, renal failure is especially common and associated with high mortality rates among hospitalized patients [8]. Acute kidney injury (AKI) has been reported in 24% to 57% of hospitalized patients and in 61% to 78% of those in the intensive care units [9–12]. COVID-19 patients who develop severe AKI, have greater dialysis requirements, experience less in-hospital recovery [10] and have a higher risk to develop chronic kidney disease [13]. Most patients with COVID-19-related AKI who recover continue to have low renal function after discharge from the hospital [14].

SARS-CoV-2 RNA has not been detected in the kidneys of COVID-19 patients [15], and it is likely that the renal failure is secondary to a number of mechanisms including immune dysregulation and inflammation, endothelial damage, thrombophilia, thrombotic microangiopathy, and complement activation [16–18]. Even though complement is a central driver of COVID-19 immunopathology [19–24], the pathways underlining the direct or indirect activation of the complement system in the kidney tissue have not been studied.

Systemic inflammation can trigger uncontrolled activation of complement and platelets. Additionally, tissue hypoxia can cause the destabilization of endothelial function. Several key proteins may contribute to the development of injury. In response to ischemia, mucin-1 (MUC1) levels increase and stabilize both HIF-1α and β-catenin to potentiate downstream protective pathways, while the prolonged upregulation of MUC1 in the injured kidney leads to chronic kidney disease through either of the pathways [25]. Our recent collaborative study confirmed that inhibition of spleen tyrosine kinase (Syk) using fostamatinib (a Syk inhibitor molecule- R406 or R788) reduces the expression of MUC1 in lung epithelial cells in a mouse model of acute lung injury [26]. Syk inhibition suppresses both local and remote organ injury [27,28], blocks Fc receptor signalling and reduces immune

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complex-mediated inflammation [29]. Syk, a non-receptor tyrosine kinase, is expressed on both hematopoietic cells and non-hematopoietic cells with a variety of functional effects including mitosis, differentiation, cellular adhesion, motility [30] and has a key role in adaptive immunity [31].

Several reports have provided insight into the role of Syk in COVID-19-related infection and lung injury [26,32]. Both Syk and MUC1 have a major role in the pathogenesis of disease through the hypoxia pathway [33–35]. SARS-CoV-2 causes hypoxia [36] and it is also known that hypoxia leads to modulation of calcium cell signalling [37]. Calcium/calmodulin-dependent protein kinase IV (CaMK4) depends on calcium signalling and is essential for the activation of transcription factors downstream of T-cell receptor (TCR) signalling [38,39]. Calcium signalling is essential for podocyte injury [40,41], but the role of CaMK4-dependent signalling in SARS-CoV-2 related kidney injury has not been addressed.

A single-cell atlas of postmortem kidney tissue samples from 16 patients who succumbed to COVID-19 failed to detect viral RNA in the kidney [15]. The non-viral mechanisms which account for the AKI in COVID-19 patients [10,11] remain poorly understood. In this communication we determined the deposition levels of complement components (C1q, C3, FH, C5b-9), Syk, MUC1 and CaMK4 along with immunoglobulin in the kidney tissues of people who succumbed to COVID-19.

2. Materials and methods

We included kidney tissue samples from three COVID-19 patients, two female and one male (ages 82, 58 and 75 years) who expired between April 22 and May 6, 2020 at the Beth Israel Deaconess Medical Center (BIDMC), Boston, United States. Kidney tissues were collected by minimally invasive ultrasound-guided autopsy within 3 h of death to maintain tissue viability. Detailed description of the sample acquisition technique, pathology and molecular findings have been presented previously [42]. Kidney tissues were also collected from autopsy material of two subjects, one male and one female, 65 and 59 years of age, who had expired in the pre-COVID-19 period (one who had suffered a middle cerebral artery syndrome stroke and another who had a diagnosis of Type I diabetes mellitus) to serve as controls. Family members were consented for limited autopsies by a pathologist during a witnessed phone call immediately after the death and after referral from the intensive care unit team. Research using autopsy tissue for this project was approved by the institutional review board (IRB) of BIDMC. A Health Insurance Portability and Accountability Act (HIPAA) waiver was granted by the IRB for access to the charts of the patients for each project using the tissue. Tissue was provided to research teams per previously IRB-approved research protocols.

For immunofluorescence studies, formalin-fixed paraffin-embedded tissue sections were deparaffinized, dehydrated and followed by heat-induced epitope retrieval in sodium citrate buffer (10 mM Sodium citrate, 0.05% Tween 20, pH 6.0). The sections were then blocked with 3% bovine serum albumin (BP1600–100, Thermo Fisher) and 5% goat (50-062Z, Thermo Fisher) or donkey (S30-100ML, Sigma-Aldrich) serum for 1 h and incubated with the primary antibody overnight. Next day, the sections were washed, incubated with the secondary antibody for 90 min washed again and stained with DAPI (S36973, Thermo Fisher). Primary antibodies used in this study included antibodies to complement molecules [C1q (A201), FH (A229) (all from Quidel), C5b-9 (ab55811, Abcam), C3 (204,869, Calbiochem)], CaMK4 (ab3557, Abcam), MUC1 (ab80952, Abcam), human IgG + IgM + IgA (ab8512, Abcam), Syk (626,202, Biolegend) and synaptopodin (SC21537, Santa Cruz Biotechnology) to detect podocytes. Secondary antibodies were donkey anti-mouse (A10037), donkey anti-goat (A11055), goat anti-rabbit (A11011) (all from Life Technologies), and goat anti-hamster (127,545,160, Jackson immune research) conjugated with Texas red or fluorescein. Images were captured by BZ-X800E Fluorescence Microscope, KEYENCE and analyzed by the BZ-X 800 software ver. 1.1.1.8, KEYENCE. Quantitative fluorescence intensity measurements were performed with ImageJ (version 1.53).

Numerical data are expressed as mean fluorescence intensity per cell. Analysis was performed using statistical software (Graphpad Prism, USA). Comparisons among groups were performed by Mann-Whitney *U* test or one-way ANOVA, followed by Bonferroni's multiple comparison test. Statistical significance was accepted at $p < 0.05$. Experiments were performed in triplicate or quadruplicate.

3. Results and discussion

Analysis of immunohistochemical images (Fig. 1A) obtained from sections of formalin-fixed paraffin-embedded kidney tissues demonstrated that SARS-CoV-2 infection instigated the deposition of C1q of the classical pathway, C3, the central molecule of all pathways, and C5b-9, the membrane attack complex. We observed increased C1q and C3 deposition in tubules and glomeruli in all COVID-19 samples. C5b-9 deposition was absent in control kidney tissues and was primarily deposited in tubular basement membranes of COVID-19 samples (Fig. 1A, Supplementary Fig. 1). In accordance with our previous study [20] in COVID-19 infected lung tissues, we observed reduced expression of complement factor H (FH), a key inhibitor of the activation of the alternative complement pathway, in the kidney samples of COVID-19 patients compared with the control tissues. In addition, we found immunoglobulin deposited at increased levels in the kidney tissues of individuals with COVID-19 (Fig. 1A). Quantitative image analysis of kidney tissues further confirmed significantly higher deposition of the C1q, C3 and C5b-9 and total immunoglobulin with decreased expression of FH (Fig. 1B).

Complement activation has been associated with ischemia, trauma and sepsis and is linked with disease severity and poor clinical outcome [43–46]. Activation of the complement system in patients with SARS-CoV-2 infection may initiate major complications in the kidney that lead to AKI. Although, clinical evidence has demonstrated increased prevalence of AKI in patients with SARS-CoV-2 infection [9–12], SARS–CoV-2 virus was not detected in the kidney tissues that were collected for this study [42]. In agreement, previous studies also did not report robust signals of SARS-CoV-2 mRNA copies in the kidneys of COVID-19 patients [15,20,47–49].

The results of this work confirmed that infection with COVID-19 induces the deposition of C1q and C3 in kidney tissue that clearly implies the activation of the classical complement pathway, and this is further supported by the enhanced deposition of immunoglobulin. It is not yet clear whether the deposited immunoglobulin represents specific antibody against SARS-CoV-2 or naturally occurring antibodies which are present in normal individuals and are known to be deposited to injured tissues [50]. A recent study revealed the generation of autoantibodies in people with COVID-19 [51] which may be deposited in tissues and activate complement. Our finding revealed, like in the lung tissues infected with SARS-CoV-2 [52], decreased deposition of the regulatory complement component FH in the kidney tissues of COVID-19 patients, which may have enabled uncontrolled complement activation and deposition. Recently, variants of the *FH* gene were reported to be associated with severe clinical outcome in COVID-19 patients [53]. This implies that certain individuals have limited ability to control complement activation and accordingly they are more susceptible to severe disease. In another study complement *FH* gene polymorphisms and haplotypes were reported to provide protection against severe dengue virus-caused disease [54]. We also noted deposition of C5b–9 in tubular cells which is apparently responsible for cell destruction.

A number of molecules have been shown to be involved in organ injury including Syk [27,28], MUC1 [25,55,56] and CaMK4 [40,41,57]. Accordingly, we determined the expression levels of these molecules in kidney tissues from patients with COVID-19. Immunohistochemical images and quantitative image analysis established the extensive

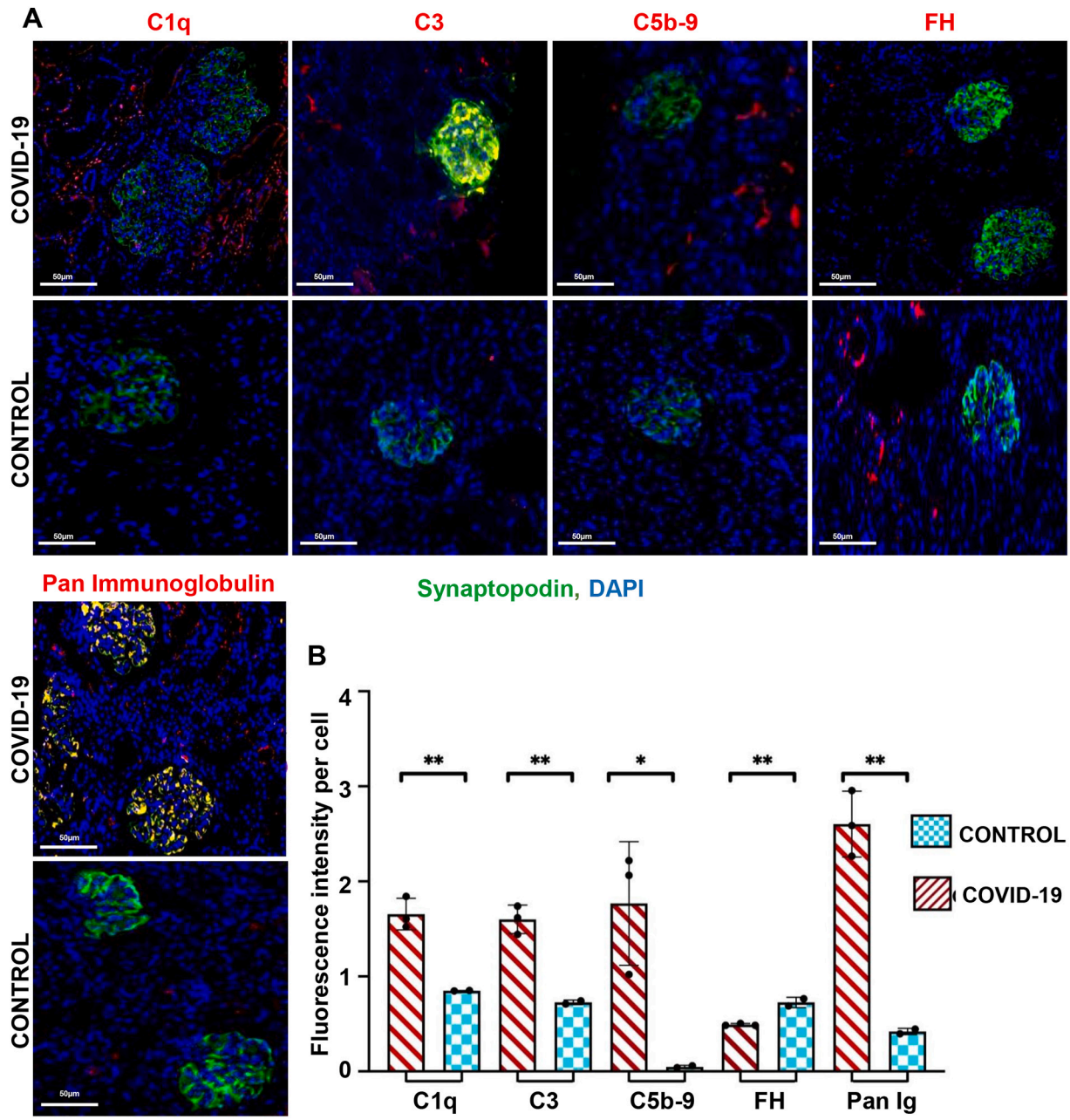

Fig. 1. Deposition of complement components and immunoglobulin in the kidneys of COVID-19 patients. (A). Representative immunofluorescence staining (merged images) of kidney sections for complement molecules (C1q, C3, C5b-9 and FH), pan-immunoglobulin (all red) and synaptopodin (green) for podocyte detection. Nuclei are stained with DAPI (blue). Monochromatic (red) images of the studied proteins in COVID-19 samples are shown in Supplementary Fig. 1. *Scale bar, 50 μm.* (B). Mean fluorescence intensity per cell in COVID-19 and control samples (*Statistical significance was accepted at $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

expression of Syk, MUC1 and CaMK4 in the kidney tissues of all three COVID-19 patients compared to controls (Fig. 2A and B).

Syk actively participates in many cell processes including adhesion, platelet activation, and innate immune recognition. These biological processes in which Syk participates has made Syk a convincing target for therapeutic strategies and approximately 70 patents have been filed using molecules to inhibit Syk for the treatment of a multitude of diseases [58]. Previously, our laboratory demonstrated that inhibition of Syk activity by the Syk inhibitor fostamatinib abolished intestinal (local) and lung (remote) tissue damage after mesenteric ischemia reperfusion [27]. Fostamatinib was approved in April 2018 by the FDA for the treatment of chronic immune thrombocytopenia. Moreover, fostamatinib is effective in limiting kidney injury in experimental animal models of glomerulonephritis [59], vasculitis [60] and proliferative immunoglobulin A (IgA) nephropathy [61]. Syk inhibition effectively inhibits IgE- and IgG-mediated activation of Fc receptor signalling and reduces immune complex-mediated inflammation [29]. Syk is also required for pathogen engulfment in complement-mediated phagocytosis [62]. These reports and our results suggest that Syk inhibitors may have a strong potential to treat COVID-19-related kidney injury.

MUC1, besides being involved in the development of tissue injury, is important in the defense against pathogen invasion [25,56]. MUC1 localizes to distal convoluted tubules and collecting ducts [63] in the kidney, however, under ischemic conditions MUC1 is localized in the proximal tubule [25,55,56]. We found higher expression of MUC1 in the kidney tissues of COVID-19 patients compared to controls. Our reported

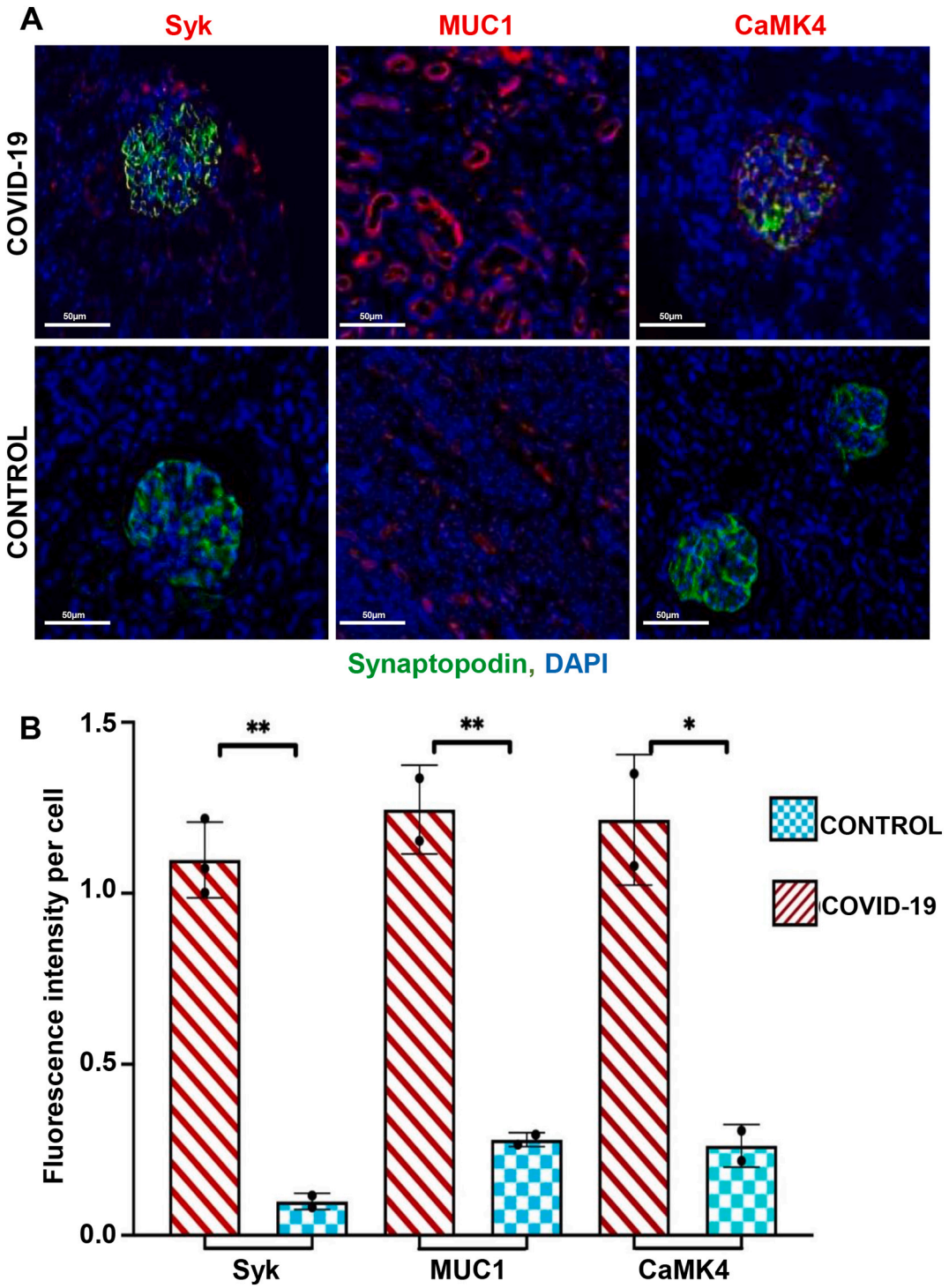

Fig. 2. Expression of CaMK4, Syk and MUC1 in the kidney of COVID-19 patients. (A). Representative immunofluorescence staining (merged images) of kidney sections for Syk, MUC1, CaMK4 (all red), synaptopodin (green). and nucleus (blue, DAPI). Monochromatic (red) images of the studied proteins in COVID-19 samples are shown in Supplementary Fig. 1. *Scale bar, 50 μm.* (B). Mean fluorescence intensity per cell in COVID-19 and control samples (*Statistical significance was accepted at $p < 0.05$). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

collaborative study showed that Syk inhibition may provide a beneficial effect in COVID-19-associatd acute lung injury by reducing the expression of MUC1 in lung epithelial cells [26]. A recent study suggested that the levels of KL-6/MUC1 may serve as a good prognostic biomarker of disease severity in hospitalized COVID-19 patients [64]. MUC1 defines both protective and harmful effects. The dual character of MUC1 has been established in many diseases including - kidney ischemia-reperfusion injury [55] and cancer metastasis [65].

Calcium signalling is involved in many pathways and regulates the expression of complement components, Syk, MUC-1 and NF-kB pathways [66–70]. In this study we found increased expression of CaMK4 in the kidney tissues of COVID-19 patients compared to controls. CaMK4 plays a role in kidney resident cell malfunction and injury including mesangial cells, podocytes and tubular epithelial cells. CaMK4 has a crucial role in controlling the structure and function of podocytes and proliferation of mesangial cells [71]. It was shown that CaMK4 hampers

liver cell viability and proliferation and drives apoptosis [72]. Our group has explored tdelivery of KN-93 to inhibit CaMK4 in a cell-targeted manner in T cells [57] and podocytes [41] in lupus-prone mice. CaMK4 inhibition prevents podocyte injury, decreases mesangial IL-6 production and prevents kidney damage and interstitial inflammation [41,57]. We propose that targeted delivery of a CaMK4 inhibitor to resident kidney cells may have a promising therapeutic potential in COVID-19 related AKI. Novel *in vitro* culture platforms of podocytes [73] may also be utilized to study the pathobiology of COVID-19 related kidney injury and drug toxicity.

In conclusion, we report increased deposition of components of the complement pathways and increased expression of Syk, MUC-1 and CaMK4 in kidney tissues of people who succumbed to COVID-19. The pattern of complement deposition indicates the activation of both classical and alternative pathways. Any of the molecular pathways, alone or in combination that participate in the interplay of these molecules, may activate the immune response in COVID-19-associated AKI. Selective inhibition of these molecules or pathways may advance the treatment strategy of COVID-19 patients. This study provides additional rationale for the expansion of clinical trials to include modulators of the expression of the involved molecules.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clim.2021.108795.

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