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Appearances Can Be Deceiving - Viral-like Inclusions in COVID-19 Negative Renal Biopsies by Electron Microscopy

Clarissa A. Cassol[1], *Neriman Gokden*[2], *Christopher P. Larsen*[1], *and Thomas D. Bourne*[1]

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Since the discovery of the causative agent for the novel severe acute respiratory syndrome (SARS)–like pneumonia syndrome pandemic that started in China in 2019 (1,2), a coronavirus named SARS coronavirus 2 (SARS-CoV-2), electron microscopy images have populated the medical literature (2) and media outlets alike displaying the characteristic 60–140 nm round particles surrounded by a "corona" of 9–12 nm distinctive spikes (2). Although many of these images were obtained after "*in vitro*" infection of cultured cells with SARS-CoV-2 (2) and are thus likely a true representation of viral particles, we have observed morphologically indistinguishable inclusions within podocytes and tubular epithelial cells both in patients negative for coronavirus disease 2019 (COVID-19) as well as in renal biopsies from the pre–COVID-19 era. Although direct infection of the kidney is theoretically possible, given the presence of angiotensin-converting enzyme 2 (the receptor used by SARS-CoV-2 to gain access to cells) within proximal tubular epithelium (3) and podocytes (4), the virus has not been detected by real-time RT-PCR in urine samples from patients with COVID-19 (5–7). Additionally, for the virus to have access to kidney parenchyma, viremia should occur, and this has only been detected in a minority of patients (6–8).

We would, therefore, like to issue a note of caution for inferring viral tissue infection by morphology alone using electron microscopy images from tissues obtained from biopsies or autopsy material in patients with COVID-19. Moreover, caution should be used when interpreting immunohistochemical results, especially within proximal tubules, which are prone to nonspecific staining by a variety of antibodies due to their intense reabsorptive capacity. Additionally, more specific techniques such as immunoelectron microscopy using specific viral antigens (9), or *in situ* hybridization for viral RNA, are likely necessary to undoubtedly confirm tissue infection in these cases.

Indeed, in two recent reports of collapsing glomerulopathy in patients with COVID-19, viral RNA was not detected in the kidney by *in situ* hybridization (10,11). Additionally, immunohistochemical analysis using a SARS-CoV-2 nucleoprotein antibody previously shown to have positive staining in the kidney of patients with COVID-19 showed nonspecific positive staining in the renal parenchyma of all kidneys in our laboratory (10).

We postulated that endogenous mimickers could be present that are morphologically indistinguishable from SARS-CoV-2 virions ultrastructurally. To confirm this, we reviewed cases under the electron microscope, looking for round cytoplasmic inclusions in podocytes, tubular epithelial cells, or endothelial cells, each with an individual diameter between 60 and 140 nm, present either in isolation or in groups. Approval for this study was obtained by the Solutions Institutional Review Board, and the ethical principles highlighted by the Declaration of Helsinki were followed. To optimize ultrastructural morphology, we excluded cases that were rapidly processed and only reviewed cases for which the renal biopsy tissue underwent routine processing (this included overnight polymerization and standard grid staining). Five cases were from the pre–COVID-19 era and ten cases were recent (biopsy dates from February to April 2020). Eight cases were allografts and six were native kidney biopsies. Case details can be found in Table 1. Viral-like inclusions, consisting both of single vesicles with diameters between 50 and 139 nm, as well as packed groups within larger vesicles, were found in all 15 cases, either in podocytes, tubular epithelium, or vascular endothelial cells (Figure 1).

Additionally, we have performed *in situ* hybridization for SARS-CoV-2 RNA in eight biopsies from patients with active COVID-19 who had evidence of kidney disease and were unable to detect virus RNA in renal tissue, despite adequate positive controls. This appears to be in contrast with a recent study that showed SARS-CoV-2 RNA could be detected by RT-PCR within renal tissue in 13 of 22 autopsied kidneys; however, it should be noted that the viral RNA levels detected were quite low (close to the lowest limit of detection of one copy per cell), and could potentially represent viral RNA within renal blood vessels (12). Microdissection by renal compartment was done in only six cases, and of those only three showed positive viral RNA within the glomerular compartment, which again could still potentially be secondary to viral

[1]Arkana Laboratories, Little Rock, Arkansas
[2]Department of Pathology, University of Arkansas for Medical Sciences, Little Rock, Arkansas

Correspondence: Dr. Clarissa A. Cassol, Arkana Laboratories, 10810 Executive Center Drive, Ste. 100 Little Rock, AR 72211. Email: clarissa.cassol@arkanalabs.com

Table 1. Biopsy characteristics including date, indication, brief clinical history, and final diagnosis

Case	Biopsy Type	Biopsy Date	Biopsy Indication	Clinical History	Biopsy Diagnosis
1	N	April 2020	Proteinuria (10 g), increased Cr (1.7)	33 yr F with DM2, CHF	Diffuse and nodular glomerulosclerosis, consistent with diabetic nephropathy, class 3
2	N	April 2020	AKI (Cr 2.7), low C4	75 yr M with HTN, hyperlipidemia, CVA, lung cavitary lesions	Mesangiopathic immune complex disease, most consistent with resolving-phase infectious glomerulonephritis
3	N	April 2020	AKI (Cr 8.4)	46 yr M with hematuria, hemoptysis, and weakness	Diffuse crescentic and necrotizing GN, pauci-immune (ANCA-associated) type
4	N	April 2020	AKI (Cr 1.5)	33 yr M with h/o methamphetamine abuse	Thrombotic microangiopathy
5	N	March 2020	Rapid decline of renal function	72 yr M with DM2, HTN on hydralazine. Positive ANA (1:640) and ANCA	Mesangiopathic immune complex disease, suspicious for autoimmune diseases such as lupus or lupus-like conditions
6	N	March 2020	History of IgAN, now nephrotic-range proteinuria	40 yr, Cr 1.3;UA: 3+ blood, 3+ protein	IgAN with focal cellular crescents and fibrinoid necrosis
7	T	March 2020	AKI (Cr 7.9)	47 yr M with ESKD from DM/HTN	Acute cellular rejection, Banff IB Acute vascular rejection, Banff IIB C4d negative SV-40 negative
8	T	March 2020	AKI (Cr 2.7)	55 yr M with ESKD from RCC requiring bilateral nephrectomies	Borderline changes by Banff criteria (suspicious for acute cellular rejection) C4d negative SV-40 negative
9	T	March 2020	AKI (Cr 4.3), anemia, leukopenia	28 yr M with ESKD of unknown etiology	Acute cellular rejection, Banff IB Acute vascular rejection, Banff IIB C4d negative SV-40 negative
10	T	February 2020	AKI (Cr 5.1)	36 yr F with h/o ESKD due to SLE. Admitted with pulmonary HTN, acute decompensated HFpEF, and AKI. DSAs negative	Negative for rejection Findings favor thrombotic microangiopathy C4d negative SV-40 negative
11	T	October 2019	AKI (Cr 6.5)	49 yr M with ESKD from DM/HTN s/p DDKT with DGF	Negative for rejection C4d negative SV-40 negative
12	T	October 2019	AKI (Cr 1.7)	53 yr with ESKD from DM2	Borderline changes by Banff criteria C4d negative SV-40 negative
13	T	October 2019	AKI (Cr 4.6)	38 yr M with HTN, AFib, HLPD, s/p kidney transplant with diarrhea	Acute cellular rejection, Banff IA C4d positive SV-40 negative
14	T	October 2019	Pain over allograft, AKI (Cr 1.8)	40 yr M with ESKD from HTN/DM	BK polyomavirus nephropathy Negative for rejection C4d negative SV-40 positive

Table 1. (Continued)

Case	Biopsy Type	Biopsy Date	Biopsy Indication	Clinical History	Biopsy Diagnosis
15	T	October 2019	AKI (Cr 2.7)	67 yr F with ESKD from DM/HTN	Borderline changes by Banff criteria C4d negative SV-40 negative

N, native kidney; Cr, creatinine (all values are in mg/dl; reference range, 0.6–1.3 mg/dl); F, female; DM2, type 2 diabetes mellitus; CHF, congestive heart failure; M, male; HTN, hypertension; CVA, cerebrovascular accident; h/o, history of; ANA, anti-nuclear antibodies; IgAN, IgA nephropathy; UA, urinalysis; T, transplant kidney; SV-40, simian virus 40; RCC, renal cell carcinoma; HFpEF, heart failure with preserved ejection fraction; DSA, donor-specific antibody; s/p, status post; DDKT, deceased donor kidney transplant; DGF, delayed graft function; AFib, atrial fibrillation; HLPD, hyperlipidemia.

particles in the blood. Spatially resolved *in situ* hybridization in one example provided shows positive SARS-CoV-2 RNA within tubular epithelium and glomeruli, but it is not clear in how many cases this was present, and whether this corresponded to intact viral particles.

A number of potential natural mimickers that can generate intracellular groups of round vesicles mimicking SARS-CoV-2 virions could be listed, the most likely being endocytic vesicles and endosomal compartment components such as microvesicular bodies containing exosomes, among others. Endocytosis leads to the formation of 60–120 nm vesicles, which is within the size range described for SARS-CoV-2 (60–140 nm) (2). These endocytic vesicles may be coated by different proteins, one of the most common being clathrin (13). The presence of coating proteins may be responsible for the presence of an electron-dense area surrounding these vesicles, giving the appearance of a viral corona. The presence of clathrin-mediated endocytosis is well described in proximal tubule cells. Podocytes also rely on both clathrin-mediated as well as clathrin-independent endocytic processes to maintain the filtration barrier by regulating the uptake of integrins and lipoproteins (14). During podocyte development or after injury, the clathrin-independent pathway of raft-mediated endocytosis of nephrin and podocin has been shown to be important for proper slit diaphragm spatiotemporal orientation (14). Given its role in nephrin and podocin trafficking and distribution, it is possible that the formation of endocytic vesicles is increased in proteinuric states that are associated with loss of filtration barrier function and podocyte cytoskeletal and basement membrane remodeling. Indeed, Farquahr *et al.* (15) in their seminal studies of the glomerular ultrastructure, in the 1950s, described an increased number of cytoplasmic vesicles in children with nephrotic syndrome. Moreover, albumin endocytosis by podocytes has been demonstrated, *in vitro* and *in vivo*, in a mouse model of puromycin-induced nephrotic syndrome (16) and could contribute to an increased number of cytoplasmic vesicles in albuminuric diseases.

Proteinuria is a common finding in COVID-19, and has been described in up to 63% of patients at some point during the disease course (17). Moreover, the development of kidney injury in patients with COVID-19 has been associated with increased in-hospital mortality (18). Therefore, it is possible that autopsy series are enriched with those patients, which thus increases the probability of finding endocytic vesicles due to podocyte injury; however, specific data regarding proteinuria was mostly not available in the largest series reported to date which focused on renal pathologic findings (19). Proximal tubular cells also strongly rely on endocytic processes to fulfill their function of reabsorbing filtered macromolecules, which can be accomplished both through receptor-mediated endocytosis as well as fluid-phase endocytosis (20).

Alternatively, the viral-like inclusions could represent microvesicular bodies containing exosomes before their release onto the cell surface. Exosomes form within the endosomal compartment as intraluminal vesicles within microvesicular bodies, which eventually fuse with the plasma membrane and are released (21). Virtually all segments of the kidney in contact with the urinary space can give rise to exosomes, including podocytes (22). Recently, a model of albumin transcytosis has been proposed, through which albumin is initially endocytosed at the capillary aspect of the podocyte and subsequently exocytosed through the apical podocyte membrane within exosomes that can be detected in the urine (16). The podocytic origin of these exosomes is confirmed by the presence of proteins typical of the podocyte cell body, such as podocalyxin (16). This again could contribute to increased numbers of cytoplasmic vesicles within podocytes in patients with COVID-19 who are proteinuric, and could lead to the mistaken assumption that these represent virions. Individual exosome sizes vary, but they are generally between 30 and 150 nm (21), which falls within the size range reported for SARS coronaviruses (9). The potential for confusion of coronavirus particles with normal cellular components was in fact highlighted in a detailed ultrastructural study by the Centers for Disease Control and Prevention (CDC) of the SARS-CoV responsible for the 2003 SARS outbreak (9). The authors recommended that, in clinical specimens, the viral nature of inclusions should be confirmed by immunoelectron microscopy for viral antigens or ultrastructural viral RNA *in situ* hybridization (9).

Recognition of this pitfall of "viral-like particles" actually dates back to the 1970s, when the potential for mistakenly assuming that normal cellular components, such as phagocytic vacuoles, microvesicular bodies, or extracellular breakdown products, could represent viral particles was emphasized after a proliferation of studies claiming to have found ultrastructural viral particles within different types of cancer cells and fluids (23). Thus, we would like to echo the CDC (9) and earlier authors' observations (15,23) and issue a note of

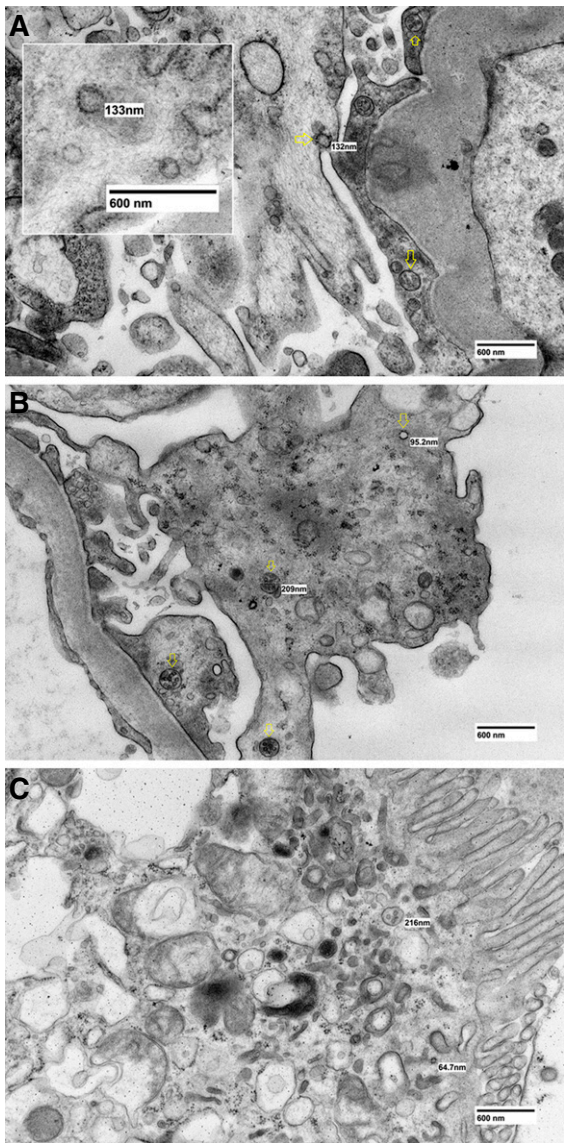

Figure 1. | Viral-like particles in non-COVID19 patients biopsies. Electron microscopy images of viral-like particles within podocytes in a case of thrombotic microangiopathy in a (A) native kidney biopsy specimen and (B) acute cellular rejection in an allograft. Note the presence in both cases of single vesicles with an electron-dense rim likely representing endocytic coated vesicles, as well as larger multivesicular bodies (arrows), which could be confounded with vesicle packets containing virions. Inset in (A): the individual small coated pits in the exterior of the vesicle bear resemblance to a viral corona. (C) Similar intracytoplasmic vesicles within tubules in an allograft with changes suspicious for acute cellular rejection.

caution regarding the use of ultrastructural images as evidence of SARS-CoV-2 tissue infection without confirmatory evidence of viral proteins or RNA in the tissue through immunoelectron microscopy or *in situ* hybridization.

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Author Contributions

T. Bourne was responsible for formal analysis and validation; T. Bourne and C. Cassol provided supervision; T. Bourne, C. Cassol, and C. Larsen conceptualized the study; T. Bourne and N. Gokden were responsible for data curation; C. Cassol was responsible for project administration and wrote the original draft; C. Larsen was responsible for resources; and all authors reviewed and edited the manuscript.

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