Kidney360 Publish Ahead of Print, published on June 30, 2020 as doi:10.34067/KID.0002692020

Appearances can be deceiving - viral-like inclusions in COVID-19 negative renal biopsies by electron microscopy

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Since the discovery of the causative agent for the novel SARS-like pneumonia syndrome pandemics that started in China in 2019[1,2] , a coronavirus named 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]. While many of these images were obtained after "in vitro" infection of cultured cells with Sars-CoV2[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 COVID-19 negative patients, 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 ACE2, 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 reverse transcriptase polymerase chain reaction (rRT-PCR) in urine samples from patients with COVID-19[5-7]. Additionally, in order 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 non-specific 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 COVID-19 patients 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 that, we reviewed cases under the electron microscope looking for round cytoplasmic inclusions in podocytes, tubular epithelial 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. In order 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 10 cases were recent (biopsy dates from February to April 2020). Eight cases were allograft 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 50-139 nm, as well as packed groups within larger vesicles, were found in all fifteen 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 8 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 levels detected were quite low (close to the lowest limit of detection of 1 copy per cell), and could potentially represent viral RNA within renal blood vessels[12].

Microdissection by renal compartment was done in only 6 cases, and of those only 3 showed positive viral RNA within the glomerular compartment, which again could still potentially be secondary to viral particles in the blood. Spatially resolved in-situ hybridization in one example provided shows positive SARS-CoV2 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, a clathrin-independent pathway, 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 in her seminal studies of the glomerular ultrastructure, in the 1950s, described an increased number of cytoplasmic vesicles in children with nephrotic syndrome[15]. 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]. It is therefore 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 focusing on renal pathological 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 prior to 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 proteinuric COVID19 patients, and could lead to the mistaken assumption that these represent virions. Individual exosome sizes vary, but are generally between 30-150 nm[21], which falls within

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 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 "viral-like" particles pitfall 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, following 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 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.

Disclosures:

C Larsen reports grants from NIH during the conduct of the study outside the submitted work. All remaining authors have nothing to disclose.

Author Contributions:

C Cassol: Conceptualization; Project administration; Supervision; Writing - original draft; Writing - review and editing

N Gokden: Data curation; Writing - review and editing

C Larsen: Conceptualization; Resources; Writing - review and editing

T Bourne: Conceptualization; Data curation; Formal analysis; Supervision; Validation; Writing - review and editing

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Table 1.

	Biopsy Type	Biopsy date	Biopsy Indication	Clinical history	Biopsy diagnosis
1	Native (N)	April 2020	Proteinuria (10g), increased Cr (1.7)	33 yrs F with DM2, CHF	- Diffuse and nodular glomerulosclerosis, consistent with diabetic nephropathy, Class III
2	N	April 2020	AKI (Cr 2.7), low C4	75 yrs M with HTN, hyperlipidemia, CVA, lung cavitary lesions	- Mesangiopathic immune complex disease, most consistent with resolving phase infectious glomerunephritis
3	N	April 2020	AKI (Cr 8.4)	46 yrs M with hematuria, hemoptysis and weakness	- Diffuse crescentic and necrotizing glomerulonephritis, pauci-immune (ANCA associated) type
4	N	April 2020	AKI (Cr 1.5)	33 yrs M with h/o methamphetamine abuse,	-Thrombotic microangiopathy
5	N	March 2020	Rapid decline of renal function	72 yrs 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 year old. Cr 1.3, UA: 3+ blood, 3+ protein	- IgAN with focal cellular crescents and fibrinoid necrosis, Oxford score M1E1S1T0
7	Transplant (T)	March 2020	AKI (Cr 7.9)	47 yrs M with ESRD 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 yrs M with ESRD 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 yrs M with ESRD 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 yrs F with h/o ESRD 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 yrs M with ESRD 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 yrs with ESRD from DM2	- Borderline changes by Banff criteria - C4d negative - SV-40 negative
13	T	October 2019	AKI (Cr 4.6)	38 yrs M with HTN, AFib, HLPD, s/p kidney transplant with diarrhea	- Acute cellular rejection, Banff IA - C4d positive - SV-40 negative

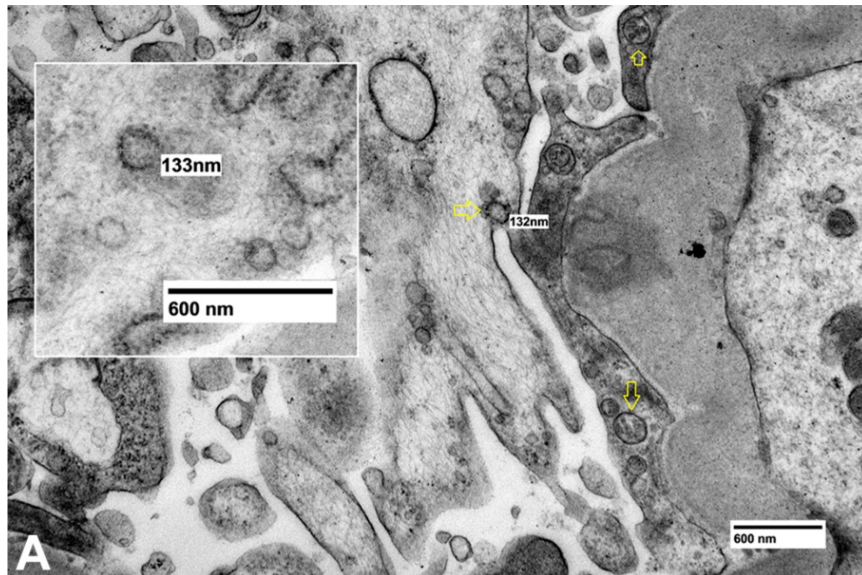
14	T	October 2019	Pain over allograft, AKI (Cr 1.8)	40 yrs M with ESRD from HTN/DM	- BK-polyomavirus nephropathy - Negative for rejection - C4d negative - SV-40 positive
15	T	October 2019	AKI (Cr 2.7)	67 yrs F with ESRD from DM/HTN	- Borderline changes by Banff criteria - C4d negative - SV-40 negative

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

Abbreviations: AKI - acute kidney injury; ANA - anti-nuclear antibodies; ANCA - anti-neutrophil cytoplasmic antibodies; CHF - congestive heart failure; Cr - Creatinine (all values are in mg/dl; reference range - 0.6 - 1.3 mg/dl); CVA - cerebrovascular accident; DDKT - deceased donor kidney transplant; DGF - delayed graft function; DM2 - type 2 diabetes mellitus; DSAs - donor specific antibodies; ESRD - end-stage renal disease; F – female; f/u - follow up; g – grams; h/o - history of; HFpEF - heart failure with preserved ejection fraction; HLPD – hyperlipidemia; HTN – hypertension; IgA - IgA nephropathy; M – male; N - native kidney; s/p - status post; SLE - systemic lupus erythematosus; SV-40 - simian virus 40; T - transplant kidney; UA – urinalysis; yrs – years.

FIGURE LEGEND:

Figure 1. Electron microscopy images of viral-like particles within podocytes in a case of thrombotic microangiopathy in a native kidney biopsy (A) and acute cellular rejection in an allograft (B). 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, 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.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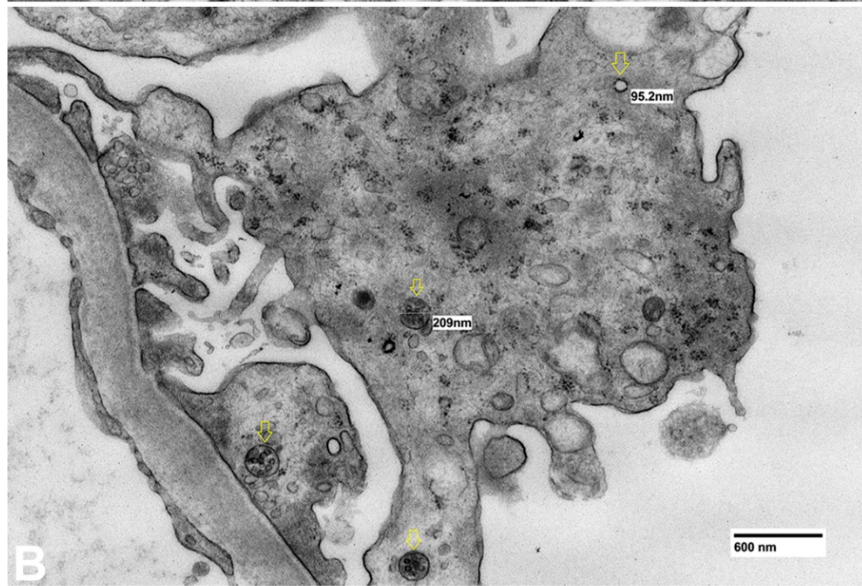
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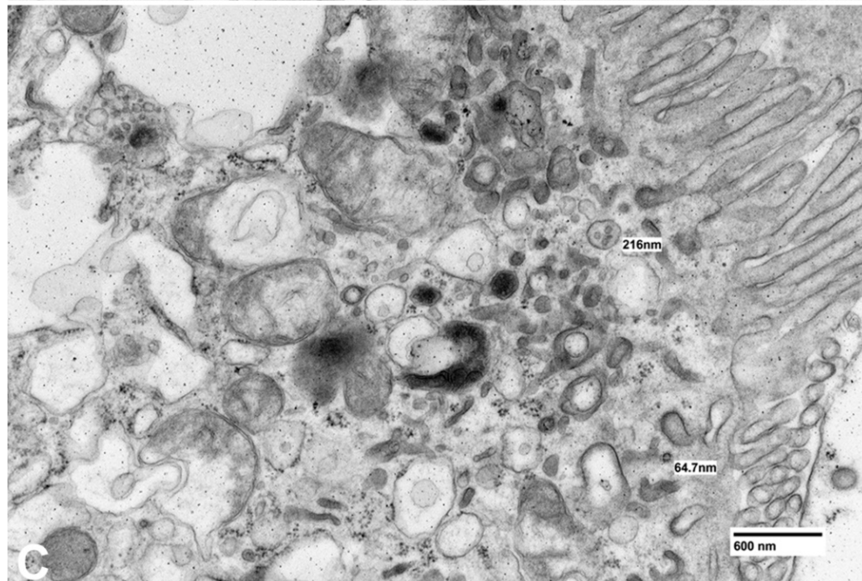
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