Arcane apoptosis in appendicitis: clue to COVID-19 in children or coincidence?

We had observed increased apoptosis in two appendixes in children who developed acute appendicitis after COVID-19 infection. We wondered if this was a chance finding or was causal. To investigate whether increased apoptosis was indicative of COVID-19 infection and if the appendix was involved in COVID-19, we evaluated H&E-stained slides of 12 consecutive cases of acute appendicitis in children (6 with past/current COVID-19, 6 negative on PCR). SARS-CoV-2 immunohistochemistry was performed on sections on the Leica BOND-III platform using Severe Acute Respiratory Syndrome (SARS) nucleocapsid mouse monoclonal antibody at 1:100 dilution (catalogue #MA1-7404; Thermo Fisher Scientific) following antigen retrieval, as previously described.[1] To determine apoptosis, we used immunofluorescence-based labelling with rabbit polyclonal caspase-3 (1:500 dilution; ab4051, Abcam UK) and visualised the localised protein using a laser scanning confocal microscope (DMi8, Leica Microsystems, Germany), as described previously.[2] Positivity was graded based on intensity and number as 0 to 3. Apoptotic cells were semiquantitatively assessed and graded from 1 to 3+ (at 20×): 0: no immunofluorescence-positive cells; 1+: less than or equal to 5 cells/high-power-field; 2+: between 6 and 10 cells/high-power-field; 3+: more than 10 cells/high-power-field.

There were three boys and three girls in the COVID-19 group (one toddler, five children (early to middle childhood); cases 1–6 in table 1). One patient was positive for SARS-CoV-2 by PCR, while the other five were negative by PCR but reactive to COVID-19 IgG antibodies in serum, indicating prior infection. In the COVID-19-negative arm, there were six children (one early childhood, one adolescent, four middle childhood; cases 7–12 in table 1).

All 12 appendixes showed dense transmural infiltrates of neutrophils. One appendix also showed eggs of *Enterobius vermicularis*, eosinophils and a granulomatous response in the muscularis propria.[3] Lymphoid follicles were present in the wall of the appendix in all 12 cases.

In the COVID-19-positive cases, apoptosis was seen in the muscularis propria and subserosa in case 1, transmurally in cases 2, 3 and 4, only in the subserosa in case 5, and in the mucosa and lymphoid follicles in case 6. The apoptosis was particularly striking in case 2 (figure 1). In the COVID-19-negative cases, apoptosis was noted in the mucosa and muscularis propria in case 7, muscularis propria only in case 8, subserosa only in cases 9 and 11, muscularis propria and subserosa in case 10, and mucosa and submucosa in case 12 (table 1). The apoptotic debris was marginally less compared with the COVID-19-positive cases.

All 12 cases were negative for SARS-CoV-2 on immunohistochemistry.

There was increased apoptosis in the appendix seen in all COVID-19 cases. The apoptotic cells were highlighted by caspase-3 immunolabelling and were predominantly inflammatory infiltrates in the lamina propria and lymphoid aggregates in the muscularis propria, occasional labelling in the epithelium, and scattered cells in the subserosa and around the adipocytes (table 1, figures 2 and 3). The COVID-19-negative cases also showed apoptosis, but less in intensity and number compared with the COVID-19-positive arm.

The ACE2 receptor is the receptor for SARS-CoV-2 and has been described in cells in multiple organs, including the intestines.[4] Within the intestines, the enterocytes contain ACE2 receptors. The organs consistently shown not to harbour ACE2 receptors include the lymph nodes, spleen, thymus, bone marrow and many cells of the immune system. The appendix has not specifically been named, but it is reasonable to assume that the enterocytes of the appendix, but not the lymphoid cells, will possess ACE2 receptors.

Caspases belong to the family of cysteinyl aspartate-specific proteases, which are highly conserved in multicellular organisms and modulate the process of apoptosis. Glutamine starvation induces apoptosis in enterocytes, and in rat intestinal epithelial cells it selectively activates specific caspases (caspases 2 and 3), leading to apoptosis. Inhibiting the caspases obstructs apoptosis, hinting at the inherent potential of caspases in attenuating apoptosis in the gut.[5]

There is a single report on the detection of SARS-CoV-2 RNA in the appendix.[6] The appendix showed granulomas, but there is no mention of the lymphoid follicles. Depletion of the lymphoid tissue in the lymph nodes, spleen, bone marrow and thymus is reported to be characteristic of infection with COVID-19.[7] Our cases did not show depletion of the lymphoid population in the appendix.

Our impression was that there was increased apoptosis in COVID-19-associated appendicitis, which was confirmed by caspase-3 immunostaining. The minor discrepancies between the morphology and immunofluorescence are because morphology detects dead cells while caspase-3 expression is the executioner caspase (in the process of dying). However, none of our 12 cases expressed SARS-CoV-2 on immunohistochemistry.

Table 1 Characteristics of patients, histology and confocal microscopy findings

Case	Sex	COVID-19 PCR status	COVID-19 IgG	Histology, site of apoptotic cells	Subserosa (IF)	Muscularis propria (IF)	Submucosa and lymphoid follicles (IF)	Mucosa (IF)
1	Male	Negative	Reactive	Muscularis propria and subserosa	0	1+	1+	1+
2	Male	Negative	Reactive	Transmural	1+	2+	3+	2+
3	Female	Positive	Non-reactive	Transmural	0	2+	1+	1+
4	Female	Negative	Reactive	Transmural	3+	3+	3+	0
5	Female	Negative	Reactive	Subserosa	0	2+	0	3+
6	Male	Negative	Reactive	Subserosa, lymphoid follicles	2+	3+	3+	2+
7	Male	Negative	Not done	Mucosa, muscularis propria	0	0	0	1+
8	Female	Negative	Not done	Muscularis propria	2+	2+	2+	2+
9	Female	Negative	Not done	Subserosa	1+	1+	0	2+
10	Female	Negative	Non-reactive	Muscularis propria and subserosa	0	2+	1+	2+
11	Male	Negative	Non-reactive	Subserosa	0	1+	1+	2+
12	Female	Negative	Not done	Submucosa	1+	1+	1+	1+

Apoptotic cells were semiquantitatively assessed and visually graded from 1 to 3+ (at 20×). 0: no IF-positive cells; 1+: less than or equal to 5 cells/high-power field; 2+: between 6 and 10 cells/high-power field; 3+: more than 10 cells/high-power field.
IF, immunofluorescence.

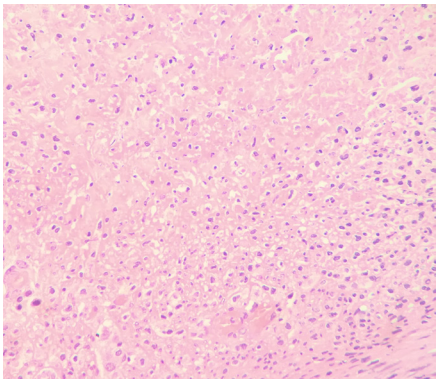

Figure 1 H&E image of case 2 showing increased apoptosis in the muscularis propria of the appendix.

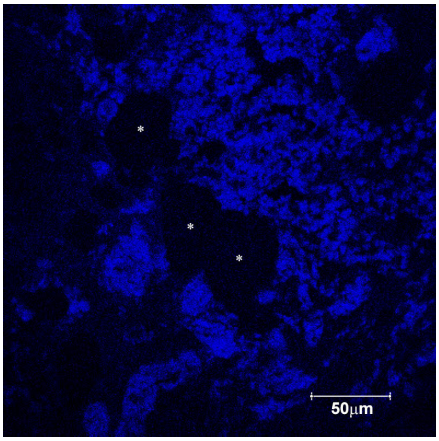

Figure 2 Low-magnification photomicrograph of caspase-3 immunoreactive cells (Cy5, blue) in the appendix captured using a DMi8 confocal microscope (Leica Microsystems, Germany). Note the profuse staining of the cells surrounding the adipose cells (*). All the cells that are stained intensely (blue) are caspase-3-immunoreactive. Scale bar: 50 µm.

Further, as apoptosis was also noted in COVID-19-negative cases, apoptosis cannot be used as a diagnostic marker of the disease.

What could the cause of the increased apoptosis be? The increased apoptosis may represent a happenstance in the two index cases or a subjective observation that did not stand up to the rigours of objective analysis. Alternatively, it could represent an inflammatory reaction to viral host response and/or a manifestation of appendiceal involvement by COVID-19. The absence of SARS-CoV-2 on immunohistochemistry argues against the last possibility. Thus, the apoptosis may be either a coincidence or secondary to viral host response.

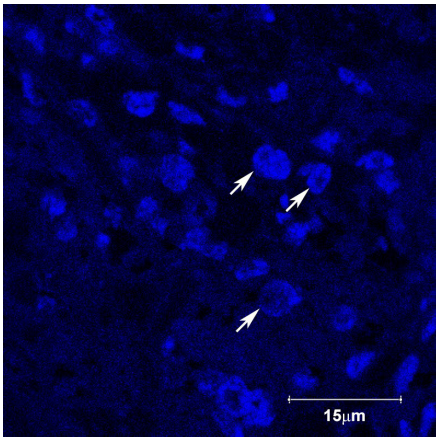

Figure 3 High-magnification photomicrograph of caspase-3 immunoreactive cells (Cy5, blue) in the mucosa of the appendix (case 2) captured using a DMi8 confocal microscope (Leica Microsystems, Germany). Note that all the cells stained intensely are caspase-3-immunoreactive. A few are identified with arrows. Scale bar: 15 µm.

Apoptosis in the appendix may be an event secondary to active infection in the upper gastrointestinal tract. Thus, lower gastrointestinal tract organs may be antibody-positive, but leave apoptotic footprints in the appendix. It may also represent an event in an immunologically overworked organ (appendix) which is not necessarily associated with active COVID-19 infection within the organ.

The appendix may be ACE2/Transmembrane serine protease-2 (TMPRSS-2) receptor-deficient/negative. Virus entry may be via endocytosis and not receptor-dependent. This is consistent with the finding by Wang *et al*,[8] who found that SARS-CoV-2 enters the cells via pH-dependent and receptor-dependent endocytosis, unlike the earlier belief that it enters the cells through direct fusion with the plasma membrane only.

In conclusion, the appendix does not express immunohistochemistry evidence of SARS-CoV-2. The association of apoptosis with COVID-19 remains purely conjunctural.

Sanjay A Pai ,[1] **Bidisha Bhaduri,**[2] **Supraja Chandrasekar,**[3] **Phalguni Anand Alladi,**[2] **Tiffany Caza,**[4] **Anita Mahadevan,**[5] **T R Saiprasad,**[6] **Patrick Walker**[4]

[1]Department of Pathology, Columbia Asia Referral Hospital, Bangalore, Karnataka, India
[2]Department of Clinical Psychopharmacology and Neurotoxicology, NIMHANS, Bangalore, Karnataka, India
[3]Paediatrics, Columbia Asia Referral Hospital, Bangalore, Karnataka, India
[4]Nephropathology, Arkana Laboratories, Little Rock, Arkansas, USA
[5]Neuropathology, NIMHANS, Bangalore, Karnataka, India
[6]Paediatric Surgery, Columbia Asia Referral Hospital, Bangalore, Karnataka, India

Correspondence to Dr Sanjay A Pai, Department of Pathology, Columbia Asia Referral Hospital, Bangalore, Karnataka, India; sanjayapai@gmail.com

Handling editor Runjan Chetty.

Contributors SAP wrote the initial draft. PW offered comments to improve the draft. SAP, TC and AM evaluated the histology slides. BB and PAA evaluated the immunofluorescence. SC and TRS offered clinical inputs. All authors have contributed to the manuscript and have seen the final version and approved of it.

Funding The authors have not declared a specific grant for this research from any funding agency in the public, commercial or not-for-profit sectors.

Competing interests TC and PW are associated with Arkana Labs, USA, where the immunostains were done.

Patient consent for publication Not required.

Ethics approval Ethics approval was obtained from the Columbia Asia Hospital Ethics Committee.

Provenance and peer review Not commissioned; internally peer reviewed.

To cite Pai SA, Bhaduri B, Chandrasekar S, *et al*. *J Clin Pathol* Epub ahead of print: [*please include* Day Month Year]. doi:10.1136/jclinpath-2021-207936

Received 20 September 2021
Accepted 14 October 2021

J Clin Pathol 2021;**0**:1–2.
doi:10.1136/jclinpath-2021-207936

ORCID iD
Sanjay A Pai http://orcid.org/0000-0002-7102-3096

REFERENCES

1 Best Rocha A, Stroberg E, Barton LM, *et al*. Detection of SARS-CoV-2 in formalin-fixed paraffin-embedded tissue sections using commercially available reagents. *Lab Invest* 2020;100:1485–9.
2 Chakraborti S, Mahadevan A, Govindan A, *et al*. Rosette-Forming glioneuronal tumor -- evidence of stem cell origin with biphenotypic differentiation. *Virchows Archiv* 2012;461:581–8.
3 Mendos A, Mathison BA, Pritt BS, *et al*. Intramural Ova of *Enterobius vermicularis* in the Appendix-An Egg-Topic Location! *Int J Surg Pathol* 2021:10668969211032455.
4 Salamanna F, Maglio M, Landini MP, *et al*. Body localization of ACE-2: on the TRAIL of the keyhole of SARS-CoV-2. *Front Med* 2020;7:594495.
5 Papaconstantinou HT, Chung DH, Zhang W, *et al*. Prevention of mucosal atrophy: role of glutamine and caspases in apoptosis in intestinal epithelial cells. *J Gastrointest Surg* 2000;4:416–23.
6 Ahmad S, Ahmed RN, Jani P, *et al*. SARS-CoV-2 isolation from an appendix. *J Surg Case Rep* 2020;2020:rjaa245.
7 Caramaschi S, Kapp ME, Miller SE, *et al*. Histopathological findings and clinicopathologic correlation in COVID-19: a systematic review. *Mod Pathol* 2021;34:1614–33.
8 Wang Y, Liu S, Liu H, *et al*. SARS-CoV-2 infection of the liver directly contributes to hepatic impairment in patients with COVID-19. *J Hepatol* 2020;73:807–16.