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CASE REPORT

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Increase of VEGF and Fibronectin expression and ultrastructural alterations of intercellular junctions in a swab negative patient after SARS-COV-2 infection

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Abstract

Background SARS-CoV-2 infection has been responsible of COrona VIrus Disease (COVID-19) pandemia and can cause a variety of symptoms including gastrointestinal disorders, abdominal pain and liver injury. The host receptor for SARS-CoV-2, ACE2, is expressed in gut and SARS-CoV-2 infection could induce vascular damage and immune system dysregulation, creating an inflammatory and hypercoagulable state, as widely described at the lung level.

Case presentation This work presents the case of a middle-aged Caucasian man admitted to the Hospital Emergency Department from the University Hospital of Ferrara (Italy), complaining of pain in the upper and middle region of the abdomen. The patient tested negative to the nose-oropharyngeal swab for SARS-CoV-2 four weeks after recovering from viral infection. The patient required resection of a segment of ileum and an ulcer of the bowel wall was recognized and sampled. Previous published results had confirmed the presence of the SARS-CoV-2 nucleocapsid protein, an increased human leukocyte antigen (HLA-G) and an altered morphology of microvilli in the ulcerated ileum of the patient when compared to the non-ulcerated ileum. The present study sought to deepen the consequences of SARS-CoV-2 infection. To this end, we evaluated the expression and co-expression of Vascular Endothelial Growth Factor (VEGF) and Fibronectin by immunohistochemical techniques. VEGF immunohistochemical expression was higher in the ulcer than in the control ileum sample and the non-ulcerated ileum areas and co-expressed with the SPIKE protein. Fibronectin staining was lower in control sample than in non-ulcerated and ulcerated ileum. Electron microscopy analysis showed alterations of the integrity of the intestinal barrier in the ulcerated area when compared to the non-ulcerated ileum or to the control sample.

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Conclusions Although the patient was tested negative to nose-oropharyngeal swab for SARS-CoV-2, the SPIKE protein was detected in his terminal ileum, especially in the ulcerated areas. The presence of the viral protein was also associated with an increase of VEGF and Fibronectin. In addition to vascular changes, the SARS-CoV-2 infection altered the junctional apparatus among epithelial cells, making the tissue even more fragile and thus susceptible to the entry of pathogens and the development of further infections.

Keywords SARS-CoV-2, Vascular endothelial growth factor, Fibronectin, Intercellular junctions, Vascular damage, Transmission electron microscopy, Immunohistochemistry, Co-expression

Background

SARS-CoV-2, responsible in early 2020 for a global pandemic, spread mainly through respiratory droplets, causing a variety of symptoms, ranging from mild respiratory difficulties to severe pneumonia and multi-organ failure, with disruption of biological mechanisms in organs and tissues [1, 2]. The physiological mechanism of gastrointestinal (GI) manifestation and following complications in SARS-CoV-2 positive patients has attracted increasing interest considering the impact of the virus in the gut tissue dysfunction, activation of cytokine storm, abdominal pain and alterations in the microbiota, strongly implicated in host immune responses to various disorders, including COVID-19 infection [3–5]. As already known, the SARS-CoV-2 protein SPIKE interacts with the cell surface enzyme Angiotensin Converting Enzyme-2 (ACE2) and the highly expressed transmembrane protease (TMPRSS2), to infect the host cell [6]. ACE2 is especially expressed in human alveolar epithelial cells but also in other organs such as stomach, small intestine and colon [7–11].

The inflammatory response triggered by the infection could disrupt the coagulation control mechanisms, with an increase in thrombosis and embolisms risk [12, 13]. In fact, respiratory tract biopsies and plasma of patients affected by SARS-CoV-2 infection reported high levels of proangiogenic factors such as Vascular Endothelial Growth Factor (VEGF), Basic Fibroblast Growth Factor (FGF-2) or Placental Growth Factors (PlGF) [14]. In particular, it has been reported that VEGF production could positively correlate with the onset of GI symptoms and COVID-19 severity, thus making it a key factor to predict disease progression [15].

At the extracellular level, both VEGF and the extracellular domain of the VEGF receptor 2 (VEGFR2) bind with the extracellular matrix glycoprotein Fibronectin (FN), that is involved in angiogenesis and coagulation processes [16]. FN is a glycoprotein involved in processes requiring reorganization of the extracellular matrix such as wound repair [17] and angiogenesis [16]. Bioinformatic analyses have identified FN as one of the top regulatory gene involved in the thrombosis process in COVID-19 patients, together also to selected miRNAs-related to the process [18]. Fibronectin has also been reported to increase in plasma from COVID-9 patients and correlate with disease severity [19, 20]. To date, no studies are reported on the variation of FN expression in the intestine. Therefore, we thought it might be of interest to evaluate this marker in our case study.

SARS-CoV-2 alterations of extracellular protein levels could affect the architecture of intestinal tissue, altering junctional structural integrity among cells and thus allowing viral penetration. As a result, an aberrant spread of pathogens into the systemic circulation occurs and induces hyperinflammation [21].

This paper reports further aspects investigated in the ileal specimen. Our previous morphological and clinical observations have shown that patient's nasopharyngeal swab negative for SARS-CoV-2 was accompanied by detection of viral nucleocapsid proteins in the GI district of the body [9] and a significant expression of HLA-G at the epithelial level, especially at the sites of highest positivity for SARS-CoV-2, was found. As previously reported, the HLA-G expression at the area of SARS-CoV-2 staining might be a cause of the viral infection dependent bleeding [9]. At the ultrastructural level, significant changes in both morphology and length of the microvilli at the apical level was demonstrated, especially in the ileal ulcerated area when compared to the non-ulcerated ileum [9]. In this study we investigated the expression of VEGF and FN markers in ileal samples of the patient apparently recovered from SARS-CoV-2 infection. Moreover, we have also examined more in depth the ultrastructural alterations of the intercellular junctions, that normally act as barrier in the intestine against pathogens and infections.

Case presentation

A Caucasian man in his fifty years of age recovered from a previous symptomatic SARS infection presented to the Hospital Emergency Department, with pain in the upper and middle region of the abdomen followed by a syncopal episode, vomiting, diarrhea and significant intestinal bleeding, as previously described in detail [9]. The patient tested negative on the nose-oropharyngeal swab for SARS-CoV-2 for the previous four weeks and had no medical history of intestinal diseases, such as Inflammatory Bowel Disease or Crohn's disease. The day after the admission, the patient underwent a colonoscopy, which revealed a small ulcer in proximity to the ileocecal

valve, and resection of the terminal ileum was necessary. Immunohistochemical analyses were performed on samples of the resected ileum within or not the ulcerated area. A pre COVID-19 ileum sample was used as control.

Figure 1 reports the immunohistochemical expression of VEGF and FN, at the level of the ileum submucosa in the three different samples, namely control ileum sample and patient non-ulcerated and ulcerated ileum samples. For each condition 4 representative sections were acquired using the NIS-Elements program by digitizing the images in TIFF format.

Imaging analysis was performed by ImageJ program (version bundled with 64-bit Java 8) using the Immunohistochemistry Profiler Plugin. The different percentages were used to calculate the H-score of each individual image [22].

The H-score values of VEGF and FN were compared by Mann-Whitney or Kruskal-Wallis tests to assess the statistically significant differences among two or three conditions, respectively.

VEGF expression resulted more evident in the submucosa of the ulcerated sample, when compared with the non-ulcerated sample. In particular, the staining was observable in proximity of capillaries and larger vascular structures. FN staining was most appreciable only at the capillary level and was more widespread in ulcerated tissue than the more localized expression of FN in non-ulcerated tissue (Fig. 1). We found that the two-marker expression was lower near the crypts in both conditions (data not shown).

VEGF staining was statistically different in the three groups ($p = 0.027$), resulting significantly higher in the ulcerated ileum sample than in the control sample and in the non-ulcerated ileum sample (Figs. 1 and 2). These data may be related to what has been seen previously: VEGF expression could be induced by the presence of the virus, possibly facilitating the onset of vascular-related tissue changes [7].

Similarly, a statistically significant difference for FN was revealed between the three groups ($p = 0.012$). FN detection was lower in the control than in the non-ulcerated and ulcerated ileum (Figs. 1 and 2).

To test the co-expression of the SPIKE virus protein with VEGF and FN, Multiplexed Immunohistochemical Consecutive Staining on Single Slide (MICSSS) technique was used. Figure 3 shows SPIKE protein, VEGF, FN and the nuclei in the control and in the patient non-ulcerated and ulcerated ileum samples.

The control ileum displays a very low expression of VEGF and FN and no immunoreaction against the SPIKE protein (Fig. 3A). The SPIKE protein was detected in both non-ulcerated and ulcerated ileum samples of the patient. The staining of the SPIKE protein, VEGF and FN in the non-ulcerated ileum resulted weak (Fig. 3B), while a more intense staining was observed in the ulcerated ileum (Fig. 3C). In addition, specific co-expressions of

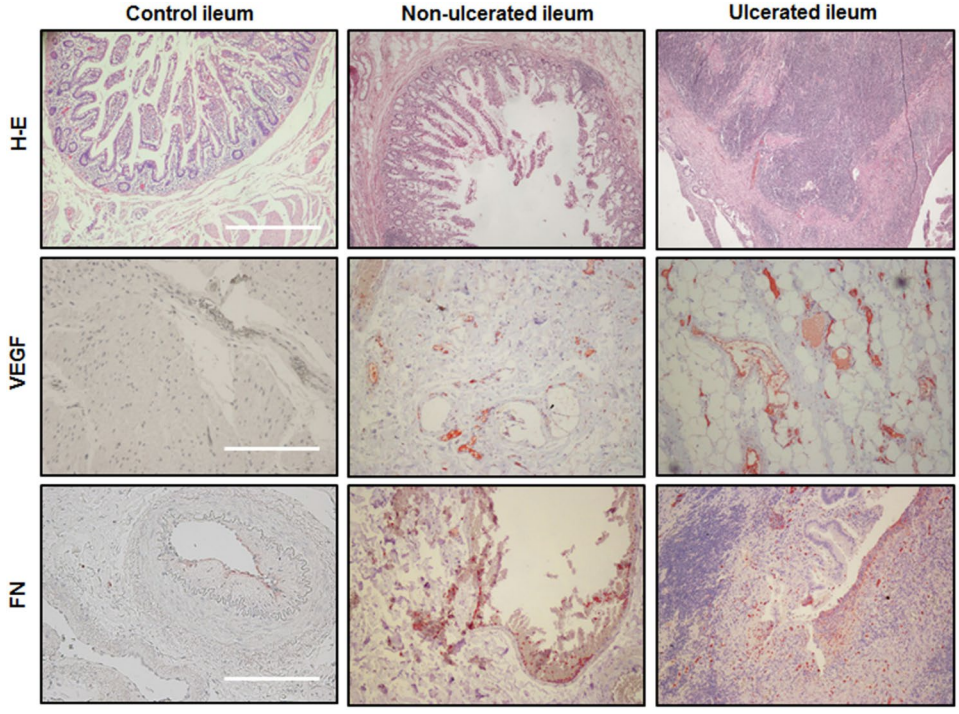

Fig. 1 VEGF and FN detection by IHC. Hematoxylin-Eosin (H-E) and immunohistochemical staining of VEGF and FN in the submucosa of control ileum and in patient non-ulcerated and ulcerated ileum. The single image is representative of four IHC ileum slices. Magnifications are 4X for H-E (bar 320 μm) and 20X for VEGF and FN (bar 100 μm)

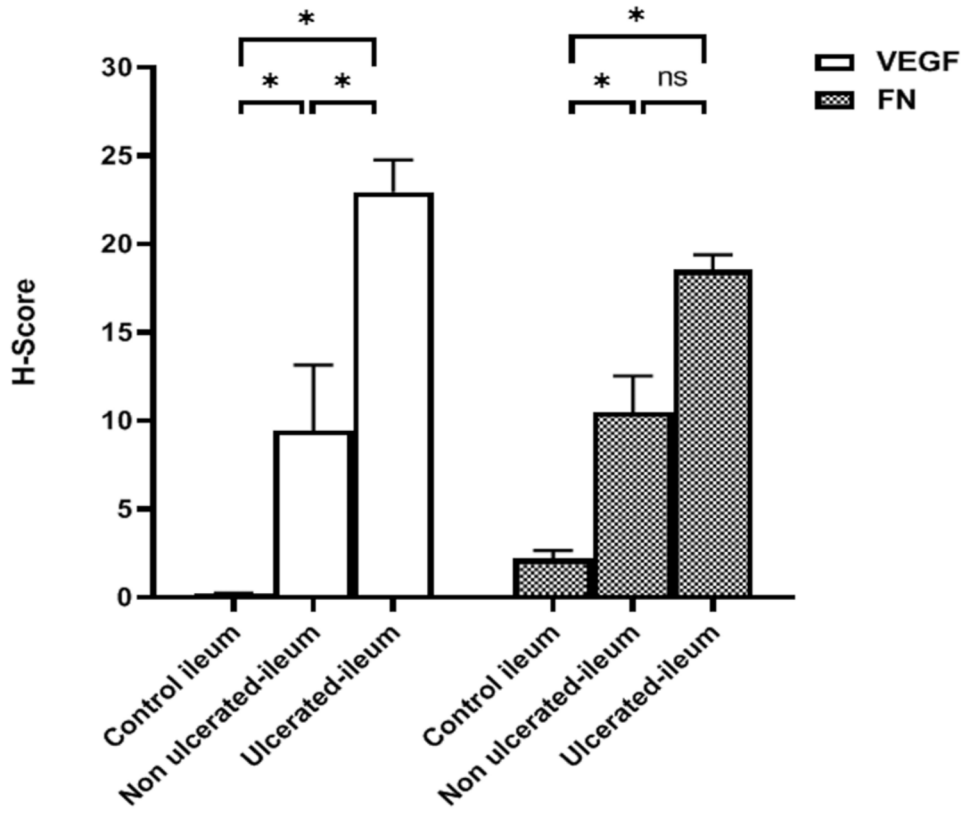

Fig. 2 H-score values of VEGF and FN in control ileum and non-ulcerated and ulcerated ileum samples. Data of markers were expressed as media ± standard deviation from four slices and compared by Mann-Whitney tests to assess the statistically significant differences of the examined markers among the specified groups. Statistical significance was set to a *p*-value < 0.05

SPIKE and VEGF, SPIKE and FN and VEGF and FN were appreciable (arrows) (Fig. 3B, C).

The SPIKE protein could be observed at the crypts level, while VEGF and in part FN were more distributed near small and large blood vessels (Fig. 3B, C).

Transmission Electron Microscopy analysis was performed to test the presence of alterations in the epithelial junctions of ileum (Fig. 4A-C). The control ileum showed linearly distributed junctions along the tissue with compact desmosomes, similarly to non-ulcerated ileum, although in the latter condition the structural aspect of the joints appears somehow less linear than in the control, as indicated by arrows (Fig. 4A, B). Instead, the junction integrity of the ulcerated tissue appeared usually altered, with the presence of intercellular spaces and structural disruptions, that may favor virus penetration deeply below the mucosal layer (Fig. 4C). We were able to observe more than 10 images presenting the altered junctions and a similar number of control images without the disrupted junction structure.

Discussion and conclusion

This expansion is crucial for a better understanding of the vascular and morphological changes induced by SARS-CoV-2, as our previous results suggested significant implications for gut health. The rationale behind this study is to elucidate the mechanisms by which SARS-CoV-2 infection leads to persistent vascular damage and immune dysregulation in the GI tract.

Previous analyses revealed, by immunohistochemistry, the presence of SARS-CoV-2 nucleocapsid protein in the patient's intestinal mucosa [9]. Therefore, we wanted to further explore the presence of specific factors or biomarkers that may correlate with vascular and coagulation impairments and that are related to viral infection. In parallel we wanted to deepen the ultrastructural analysis to assess the presence of other ultrastructural tissue changes, in addition to the previously described microvilli alterations [9], that may enhance viral penetration and diffusion inside the gut. Three months after resection of the terminal ileum the patient was referred to have a good recovery with satisfactory clinical conditions, except for a small intestinal bacterial overgrowth (SIBO), without the need of further surgical interventions.

As already known, SARS-CoV-2 infection can induce alterations of the vascular system leading to an increased risk of thrombosis [23–26]. Vascular damage is also related as a reaction to an increased level of angiogenesis [27, 28]. In fact, VEGF, the most important angiogenic molecule, has been associated with the formation of new

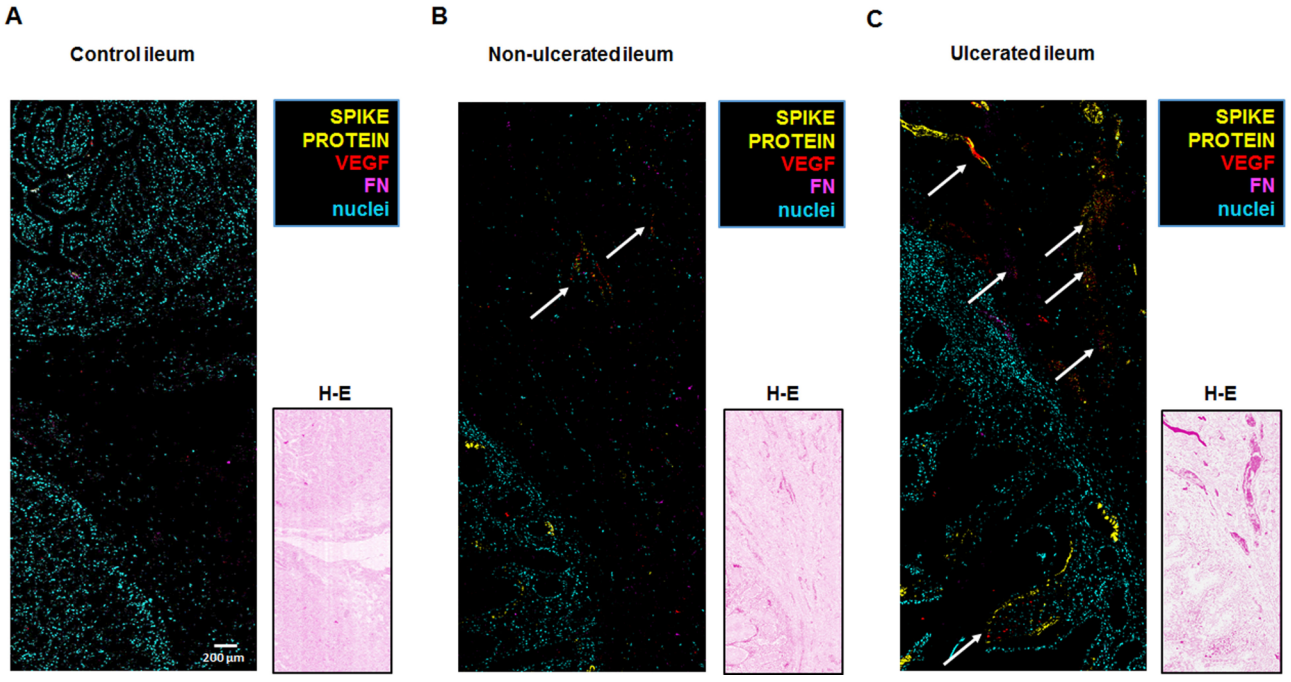

Fig. 3 Detection of SPIKE protein, VEGF and FN by MICSSS. The sections of control ileum (**A**), non-ulcerated (**B**) and ulcerated ileum (**C**) were sequentially stained and scanned for H-E, SPIKE, VEGF, FN and the nuclei. Bright-field images were inverted, and RGB channel splitting was performed. The most significant images were merged, and pseudocolors were attributed to each marker. Scale bars, 200 µm. The arrows indicate points of co-expression of the three markers analyzed in the tissue. MICSSS, Multiplexed Immunohistochemical Consecutive Staining on Single Slide

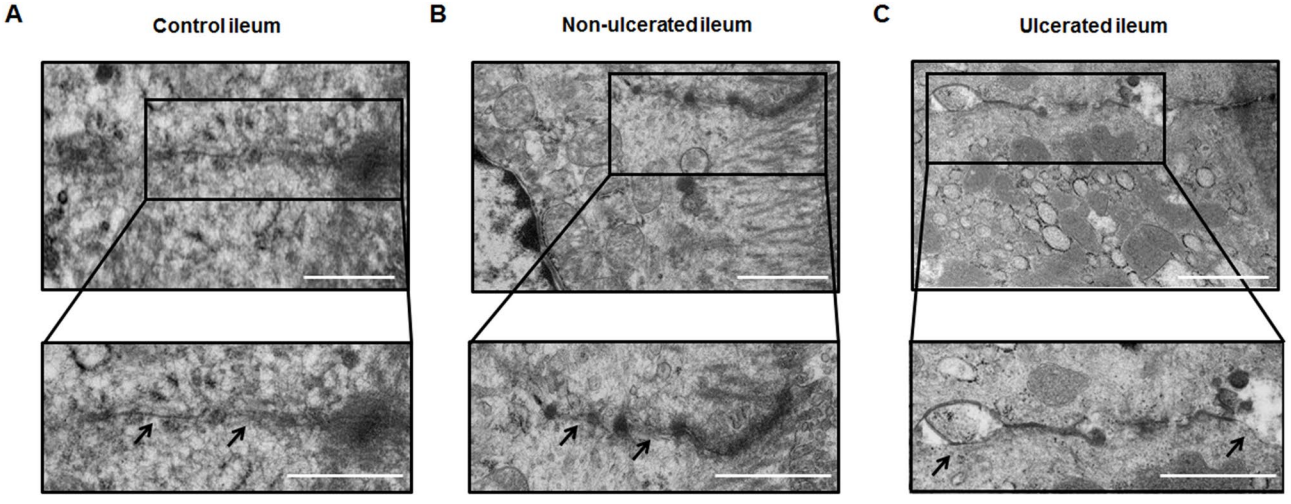

Fig. 4 Ultrastructure of intercellular junctions. Transmission Electron Microscopy showing the different morphology and integrity of intercellular junctions on Control ileum (**A**), patient ileum non-ulcerated area (**B**) and patient ileum ulcerated area (**C**). In Fig. 4A and B (smaller images) arrows indicate normal and partially altered junctions, whereas in Fig. 4C (smaller image) arrows indicate the irregular junction and the presence of intercellular spaces. TEM images are representative of a larger number (> 10) of observations of the same phenomenon. Magnifications are 11000X for the larger images (bar 1 µm) and 17500X for the smaller images (bar 500 nm)

blood vessels in gut pathologies such as inflammatory bowel disease [29], Crohn's disease [30] and ulcerative colitis [31]. In this study, VEGF increased in the patient's ileum when detected in association with the SPIKE protein. This agrees with the hypothesis that VEGF plays an important role in the increased permeability, inflammation and tissue alterations observed in GI SARS-CoV-2 complications, as reported elsewhere [15].

In parallel with the increment in VEGF expression, an increase in FN was observed in the ulcerated tissue sample.

This is agreement with the finding that in severe ill patients infected with SARS-CoV-2, FN is significantly

increased in alveolar cells, and its expression has also been analyzed to distinguish bacterial from viral infections, as SARS-CoV-2 infection [20].

At the ultrastructural level, TEM analysis revealed an important alteration of the morphology of intercellular junctions, that physiologically are one of the key elements of the barrier in the intestine to prevent pathogen entry inside the organ [21, 32–35]. In the ulcerated ileum, junctional integrity was compromised, with visible dilatations that consequently further enhanced altered permeability, as also described in other pathologies such as inflammatory bowel disease [34]. Alteration of junctional integrity following SARS-CoV-2 infection is also further documented in recent studies involving in vitro and in vivo models, with potential therapeutic strategies [36, 37].

This case report underlines how SARS-CoV-2 infection is also able to cause serious damage in other organs besides the respiratory tract, even in cases of patients who tested negative for nasopharyngeal SARS-CoV-2 [7, 9, 38]. The presence of the SPIKE protein in the patient's ileum does not confirm that the viral infection is active. However, the co-expression of the viral protein and of VEGF and FN suggests that the virus infection played a major role in the bowel alteration leading to the ileum resection. These data suggest that SARS-CoV-2 could spread to and persist in the body tissues and induce several pathological alterations. Moreover, these results agree with a very recent report that showed a relevant connection between persistent presence of residual SARS-CoV-2 and long virus infection symptoms [39].

In conclusion, in our case study we observed the pathogenicity of the SARS-CoV-2 infection in the GI tract, with a relevant virus effect at the level of vascular impairments and tight junction integrity, although the virus was no longer detected at nasopharyngeal level. Our observations support the hypothesis that SARS-CoV-2 infection can lead to significant and persistent alterations in gut morphology and function, even in patients who test negative for the virus in nasopharyngeal swabs.

Abbreviations

ACE2	Angiotensin converting enzyme 2
COVID-19	Corona virus disease 2019
FN	Fibronectin
VEGF	Vascular endothelial growth factor
HLA-G	Human leukocyte antigen
MICSSS	Multiplexed immunohistochemical consecutive staining on single slide
GI	Gastrointestinal
TMPRSS2	Transmembrane protease 2
H-E	Hematoxylin-Eosin
IHC	Immunohistochemistry
FGF-2	Basic fibroblast growth factor-2
PlGF	Placental growth factor

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Author contributions
Conceptualization: C.S., J.M.S.; methodology: C.S., J.M.S.; validation: G.C., C.S., J.M.S., R.G., S.O., L.M.N. and A.P.; software: C.S; investigation: C.S., J.M.S. and R.G.; resources: C.S.; data curation: C.S. and J.M.S.; writing original draft preparation, C.S., J.M.S.; writing review and editing, C.S., J.M.S., R.G., L.M.N. and A.P.; supervision of the work, G.C., L.M.N. and A.P.; project administration, A.P. and L.M.N. All authors read and approved the final manuscript.

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Data availability
No datasets were generated or analysed during the current study.

Declarations

Ethics approval and consent to participate
The study was conducted in accordance with the ethical principles for medical research involving human subjects as required by the 2013 revision of the Helsinki Declaration—WMA Declaration of Helsinki—The Ethical Principles for Medical Research Involving Human Subjects. The Local Ethics Committee (Comitato Etico di Area Vasta Emilia Centro della Regione Emilia-Romagna, CE-AVEC) approved this study (reference number 122/2021/Oss/AOUFe). The participant has signed informed consent to be included in the study.

Consent for publication
Not applicable. Published images cannot be linked to the patient in any way.

Competing interests
The authors declare no competing interests.

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