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ORIGINAL ARTICLE

Oral lesions with immunohistochemical evidence of Sars-CoV-2 in swab-negative post-COVID syndrome

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Abstract

Objectives: Growing evidence exists about post-COVID condition/syndrome as sequelae of Sars-CoV-2 infection in healed patients, possibly involving the lungs, brain, kidney, cardiovascular and neuromuscular system, as well the persistency of taste dysfunction. Such symptoms develop during or after infection and continue for more than 12 weeks with pathogenesis related to virus persistency but variable by organs or systems.

Materials and Methods: We recently observed six patients recovered from COVID-19 and with negative RT-PCR testing, showing oral mucosa lesions (mainly ulcers) overlapping those occurring in the acute phase, persisting up to 20 days and thus needing a biopsy with histological investigation and spike protein evaluation by immunohistochemistry.

Results: We found epithelial ulceration, inflammatory infiltrate, vessels with increased diameter and flattened endothelium but no thrombi formation; also, we found a weak epithelial SARS-CoV-2 positivity limited to the basal/spinosum layers, progressively decreasing toward the periphery, and the intraepithelial lymphomonocytes, endothelium, and perivascular pericytes too.

Conclusions: Our findings provide evidence that SARS-CoV-2 can persist, as for other organs/systems, also in the oral epithelium/mucosa after the acute phase and can be responsible for lesions, although by a pathogenetic mechanism that should be better defined but certainly referable as the oral mucosa counterpart of post-COVID syndrome.

KEYWORDS
COVID-19, immunohistochemistry, negative RT-PCR test, oral mucosa lesions, post-COVID syndrome, Sars-CoV-2

1 | INTRODUCTION

Patients with a previous diagnosis of COVID-19 or symptoms consistent with SARS-CoV-2 infection may display a wide variety of sequelae (mainly neurological and cognitive disorders, postviral fatigue, pain, cardiac, and/or pulmonary symptoms; anosmia; dysgeusia; headache) (Visco et al., 2022) for several weeks up to 24 months, generally defined as post-acute sequelae of SARS-CoV-2 or alternatively as long COVID or post-COVID condition or syndrome (PCS). The current definition of this new syndrome has been reported by the WHO as "it occurs in individuals with a history of probable or confirmed SARS-CoV-2 infection, usually 3 months from the onset of COVID-19 with symptoms and that last for at least 2 months and cannot be explained by an alternative diagnosis" (Soriano et al., 2022).

Although surely multifactorial, a truly defined pathogenetic mechanism of PCS is still missing as occurrence, targets, autoimmune response, and the significance itself of SARS-CoV-2 persistence are certainly either organ- or host-related. Indeed, PCS pathogenesis may be generally related to postviral fatigue and/or postviral recovery or to one or more of the following mechanisms, as emerging from consistent studies available in the literature: SARS-CoV-2 persistence (Chertow et al., 2021; Ramakrishnan et al., 2021; Su et al., 2022) activation of other viruses (mainly EBV), virus-triggered autoimmunity (Casanova & Abel, 2021; Su et al., 2022) persistent tissue damage and immunity-triggered inflammation (Li et al., 2022; Nalbandian et al., 2021), and virus-triggered microthrombi formation (Kell et al., 2022; Tarnawski & Ahluwalia, 2022).

Concerning the oral cavity, well-established data from literature indicate that COVID-19 patients may variably (but mainly) present gustatory dysfunction, xerostomia, and oral mucosal lesions, with prevalence and persistency likely to vary by age/sex, systemic health status, but especially SARS-CoV-2 infection stage (early or late) and disease severity (Amorim dos Santos et al., 2020; Ansari et al., 2021; Brandão et al., 2020; Chaux-Bodard et al., 2020; Favia et al., 2021; Martín Carreras-Presas et al., 2020; Tsuchiya, 2021).

Ulcers, erosions, multiple reddish maculae or blistering lesions mostly of the tongue, labial mucosa, and palate have been consistently described as oral mucosal lesions related to SARS-CoV-2 infection in the acute phase, likely for gustatory dysfunction and xerostomia, duration, and/or persistency may last variably also after infection negativity (Favia et al., 2021; Iranmanesh et al., 2020).

We recently focused the attention on patients referring a previous diagnosis of SARS-CoV-2 infection and affected by oral lesions resembling those COVID-19-related, anamnesis without additional events, negative reverse transcription-polymerase chain reaction (RT-PCR) testing at the moment of clinical investigation and lacking systemic diseases possibly responsible for the observable oral mucosa lesion. The main target in such instances has been to clarify if such post-COVID-19 lesions, strictly overlapping with several conditions not specifically related to such infection (post-intensive care syndrome, exacerbation of preexisting health conditions with oral mucosa involvement, newly developed oral lesions, etc.) could be considered the oral counterpart of the post-COVID-19 (long-lasting or not) syndrome.

2 | MATERIALS AND METHODS

This study was carried out following the principles of the Declaration of Helsinki and approved by the Independent Ethical Committee active in the University of Bari, Italy (Study No. 4652, Prot. 66/CE); patients released informed consent both for diagnostic and therapeutic procedures and for the possible use of the biologic samples for research purposes.

Patients included in the current study followed the following inclusion criteria: history of SARS-CoV-2 acute infection confirmed by RT-PCR after nasal and oropharyngeal swab occurred at least 12 weeks before the onset of the oral lesions; patients previously affected by mild/moderate forms of COVID-19 referred to the Complex Operating Unit of Dentistry of the University/Hospital of Bari-Italy from September 2021 to September 2022; patients with certain and already confirmed preexisting lesions, which means all those that were symptomatic of preexisting systemic and local conditions previously diagnosed or well-known to patients, as well as patients with traumatic lesions, patients with oral lesions during SARS-CoV-2 acute infection, were excluded; and persistency of at least 20 days of the oral lesion thus requiring an incisional bioptic sampling for diagnosis; diagnosis of long COVID received in a specialistic setting. Because of the possibility of overlap between PCS and post-intensive care syndrome, patients hospitalized in the intensive care unit were excluded too.

Patients medical history was collected including the following data: age and gender, general symptoms of COVID-19, the occurrence of oral lesions during the acute infection of SARS-CoV-2, presence of taste disorders, the time elapsed since the first negative RT-PCR test after acute COVID-19, and presence of other signs/symptoms attributable to the post-COVID condition (Table 1).

The following clinical data were collected: type of lesion and clinical remarks (site, size, and numbers), associated symptoms, quality of oral hygiene, and preexisting general/systemic conditions/diseases possibly responsible for oral mucosa lesions.

All patients underwent biopsy by local anesthesia infiltration preceded by rinsing with chlorhexidine 2% mouthwash for at least 1 min. All surgical samples were promptly fixed in 10% neutral-buffered formalin for 48 h and sent to the Pathological Anatomy Unit of the University of Bari "Aldo Moro," then embedded in paraffin, sectioned at 4 mm thickness, and stained with hematoxylin and eosin (H&E). For immunohistochemical staining, histological sections, collected on poly-L-lysine-coated slides (Sigma Chemical), were deparaffinized. The sections were rehydrated in a xylene-graded alcohol scale and then rinsed for 10 min in 0.1 M PBS. Sections were pretreated with sodium citrate pH 6.1 (Dako Corporation) in Dako PT Link for antigen retrieval solution for 30 min at 98°C and then incubated with mouse monoclonal anti CD3, CD20, CD31, CD34, SMA, CD68, CD163, and SARS-CoV/SARS-CoV-2 (Anti-SARS-N Nucleocapsid Antibody), as listed in Table 2. The SARS-CoV/SARS-CoV-2 stain more precisely included: washing sections in dH_2O three times for 5 min and incubation in 3% hydrogen peroxide for 10 min; washing sections in dH_2O two times for 5 min each and, than, in wash buffer

TABLE 1 Clinical data of patients enrolled in the current study.

Patient ID	1	2	3	4	5	6
Sex	M	M	F	M	F	M
Age	9	55	25	65	37	45
Anti-COVID vaccination (yes/no)	No	Yes	Yes	Yes	Yes	Yes
No. of vaccine doses	–	3	3	2	3	2
Form of COVID-19 (mild – Mi, moderate – Mo, severe – Se, and critical – Cr)	Mi	Mo	Mi	Mi	Mi	Mo
Onset time of oral lesions[a]	11	10	10	12	9	10
Signs/symptoms of the post-COVID-19 condition	Myalgia Arthralgia	Arrhythmia Dyspnoea	Abdominal pain Nausea Brain fog	Myalgia Abdominal pain	Asthenia Urticaria	Venous thromboembolism
Oral lesions	Buccal mucosa ulceration/lower lip fissured ulcerations	Upper lip ulceration	Buccal mucosa ulceration	Vermilion ulceration	Lower lip ulceration	Buccal mucosa ulceration

[a]The onset time of oral lesions means the weeks elapsed between the first negative swab that occurred after the acute phase of COVID-19 and the occurrence of the oral lesions.

TABLE 2 Immunohistochemical panel.

Antigen	Clone	Dilution	Source	Positive control
CD3	LN10	1/200	Novocastra	Placenta of COVID-19 positive
CD20	L26	1/200	Dako	Placenta of COVID-19 positive
CD31	1A10	1/2	Dako	Placenta of COVID-19 positive
CD34	M7165	1/500	Dako	Placenta of COVID-19 positive
CD68	PG-M1	1/100	Dako	Placenta of COVID-19 positive
CD163	10D6	1/100	Biocare Medical	Placenta of COVID-19 positive
Actin (smooth muscle)	1A4	1/200	Agilent Technologies	Placenta of COVID-19 positive
SARS-CoV/SARS-CoV-2	#001	1/2000	EliteRmab	Placenta of COVID-19 positive

eight times for 1 min; blocking sections with 100–400 μL of blocking solution for 30 min at room temperature and after removing blocking solution, adding 100–400 μL primary antibody diluted in recommended antibody diluent to each section; incubation 1 h at 37°C; washing sections in wash buffer eight times for 1 min; incubation with Horseradish Peroxidase-conjugated secondary antibody at appropriate dilution in PBS for 20 min at 37°C, and washing with wash buffer eight times for 1 min; develop with Diaminobenzidine till the appropriate color then rinse the slides under tap water gently for about 1–3 min; and washing sections in dH_2O two times for 5 min and dehydrate sections in 100% ethanol two times for 2 min and in xylene two times for 5 min, mounting sections with coverslips and mounting medium. The placenta of a COVID-19-positive patient was used as the immunohistochemical positive control.

3 | RESULTS

The patients were four males and two females with an age range of 9–65 (Table 1). One patient was unvaccinated, whereas the remaining received two (two cases) and three (three cases) doses, respectively; the clinical form of COVID already suffered by patients was mild in four cases and moderate in two and the median time of occurrence of post-COVID oral lesions was 10.3 weeks (range: 9–12 weeks); clinically, oral mucosa lesions were constantly ulcers, more precisely involving the lip (upper and lower, including vermilion; Figures 1 and 2) in four cases and the buccal mucosa in the remaining two, synchronously lip and buccal mucosa only in one, always associated with rather irregular margins, pain, often bleeding due to biting trauma, hemorrhagic areas, and necrosis, and often

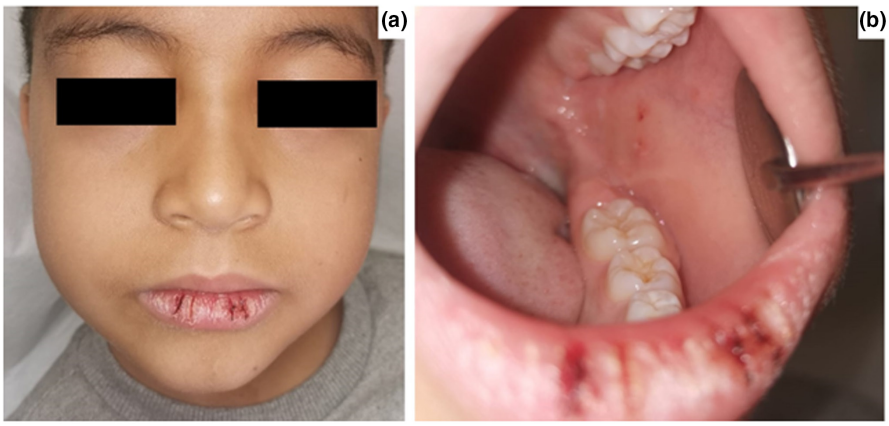

FIGURE 1 Multiple, deep, and fissuring ulcers of the lip vermillion (a) and multiple ulcers of the buccal mucosa (b) in a 9-year-old patient healed for 11 weeks from COVID-19 and with a current negative RT-PCR test, related to SARS-CoV-2 persistency demonstrated by histological examination.

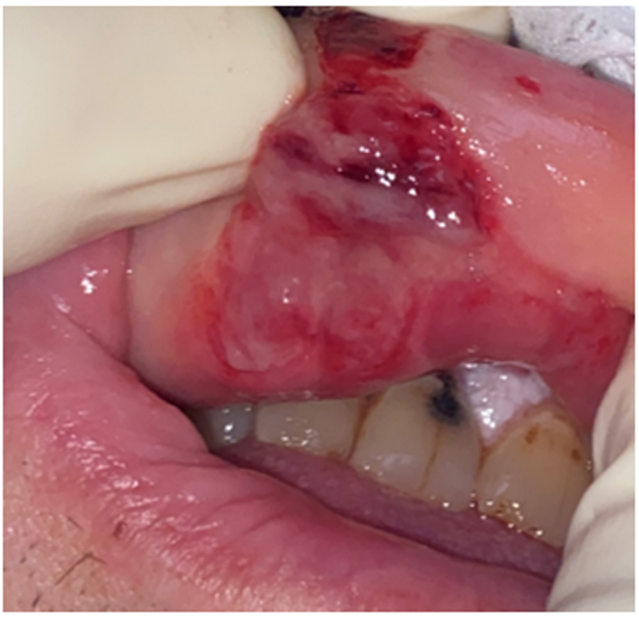

FIGURE 2 Wide and irregular ulceration of the lip mucosa with necrotic-hemorrhagic areas occurring in an adult patient 10 weeks after healing from COVID-19 and with a current negative RT-PCR test, histologically diagnosed as a lesion of the oral mucosa in the post-COVID syndrome.

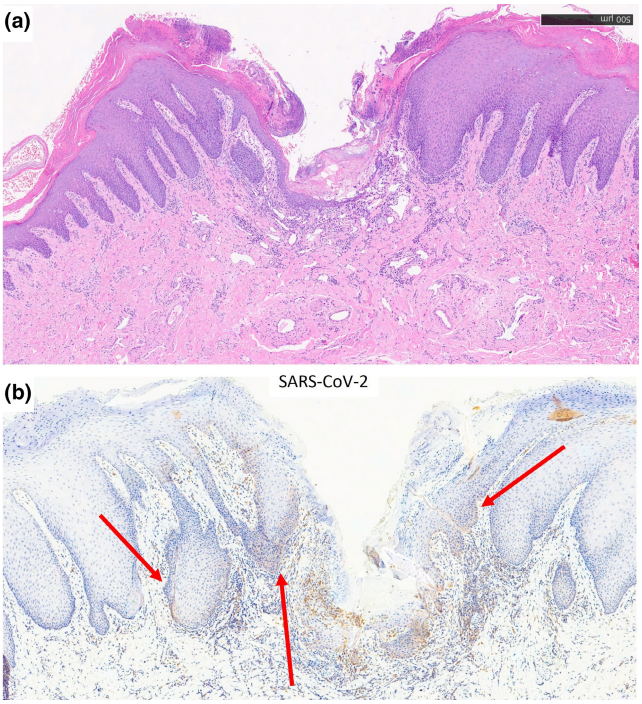

FIGURE 3 (a) Histopathological features of an erosive lesion of the lip: the epithelial lining is extremely thinned and covered by serum-fibrinous exudate, while the underlying stroma contains inflammatory cells and dilated capillary vessels; the adjacent epithelium shows marked acanthosis and orthokeratosis (H&E, 4×). (b) At low power magnification, both epithelial (weak) and inflammatory (strong) cells display immunoreactivity for SARS-CoV-2 antigens (Immunohistochemistry anti- SARS-CoV-2, 4×).

deeply involving the subepithelial tissue with increased consistency. Myalgia, arthralgia, arrhythmia, dyspnoea, abdominal pain, nausea, brain fog, myalgia, asthenia, urticaria, and venous thromboembolism were the associated signs and symptoms variably reported by patients as sequelae of the previous COVID infection. Clinical data of patients are summarized in Table 1.

On H&E stain, vacuolization of the superficial layers was inconstantly observable in the epithelium along with an inflammatory infiltrate widespread throughout the lesion; in the ulcerated areas, the epithelial lining was extremely thinned and covered by serous-fibrinous exudate; vessels generally showed increased diameter and flattened endothelium, but without thrombi formation (Figures 3a, 4a, and 5a). The epithelial layers showed a weak SARS-CoV-2 positivity of the basal and spinosum layers, progressively decreasing till absent in the uppers, whereas a strong positivity of the intraepithelial lymphomonocytes; also, such epithelial positivity was decreasing progressively from the lesion toward the periphery until completely disappearing (Figures 3b, 4b, and 5b). Immunohistochemical characterization of the inflammatory infiltrate showed consistency of CD3-positive T-lymphocytes and CD20-positive B-lymphocytes and CD68 and CD163-positive monocytes/macrophages; also, strongly positive to SARS-CoV-2, CD31 and CD34 were both the endothelium and the perivascular

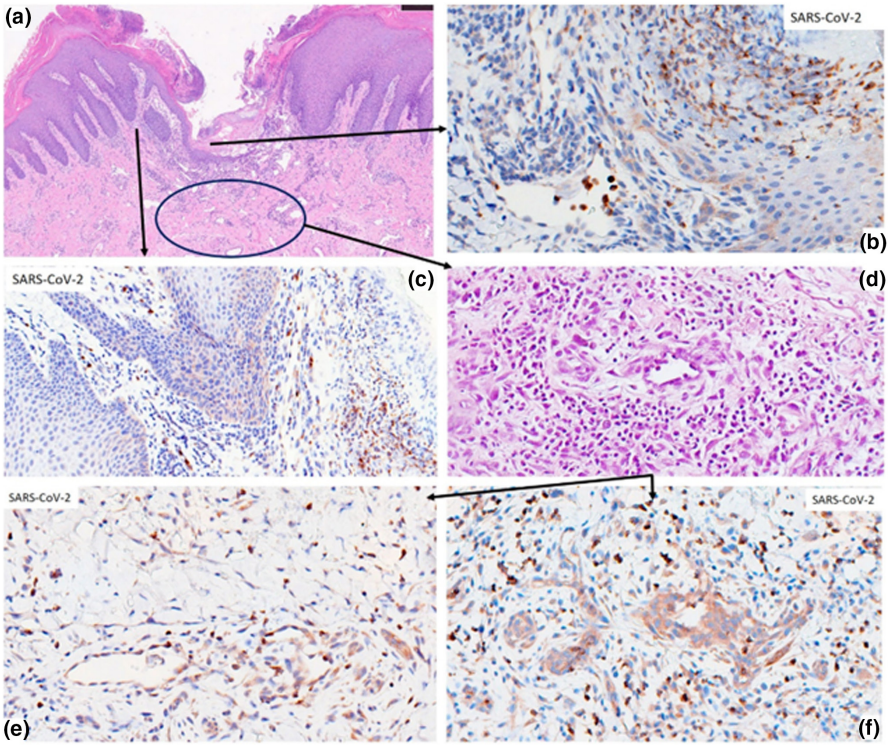

FIGURE 4 (a) Higher power view of the epithelial erosion (a – H&E, 10×) and corresponding strong immunohistochemical positivity for SARS-CoV-2 antigens in the intraepithelial lymphomonocytes (b – Immunohistochemistry anti-SARS-CoV-2, 20×) and stromal monocytes (c – Immunohistochemistry anti-SARS-CoV-2, 20×); details of a subepithelial area with inflammatory infiltration and capillary dilation (d – H&E, 20×). Both endothelial cells (e) and inflammatory lymphocytes (d) display consistent immunohistochemical positivity for SARS-CoV-2 antigens in stromal monocytes (e, f – Immunohistochemistry anti-SARS-CoV-2, 20×).

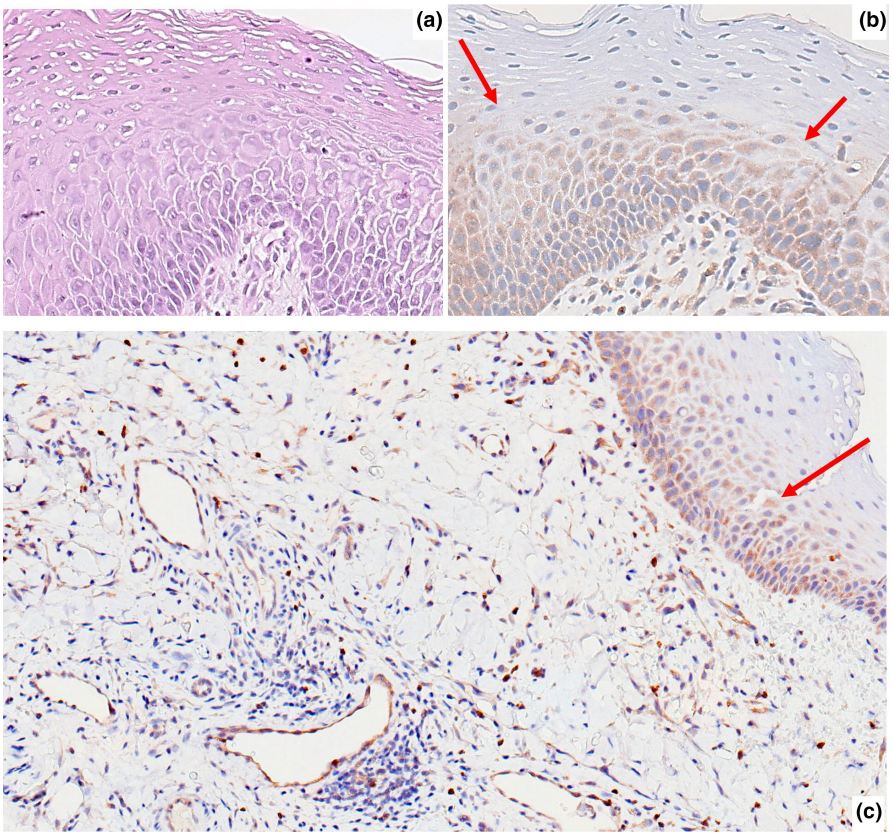

FIGURE 5 High power magnification of areas close to the principal lesion showing no epithelial ulceration and superficial vacuolization (a); at the immunohistochemistry, weak Sars-CoV-2 positivity of the basal and spinosum layers, progressively decreasing till absent in the uppers (b), and strong positivity of the endothelial cells and inflammatory infiltrate in the subepithelium.

pericytes, while the perivascular spindle-shaped cells showed positiveness to SMA too, the latter possibly related to perivascular myofibroblastic activation (Figures 4b–f and 6). Additionally, SARS-CoV-2 positiveness of the perineural infiltration was fairly constant. The positive control of the placenta of a COVID-19-positive patient is shown in Figure 7.

4 | DISCUSSION

The post-COVID-19 condition or post-COVID-19 syndrome (PCS) usually manifests about 3 months from the onset of acute illness with an estimated incidence emerging from a study on the UK population as ranging from 3% to 11.7% (Ayoubkhani & Gaughan, 2021).

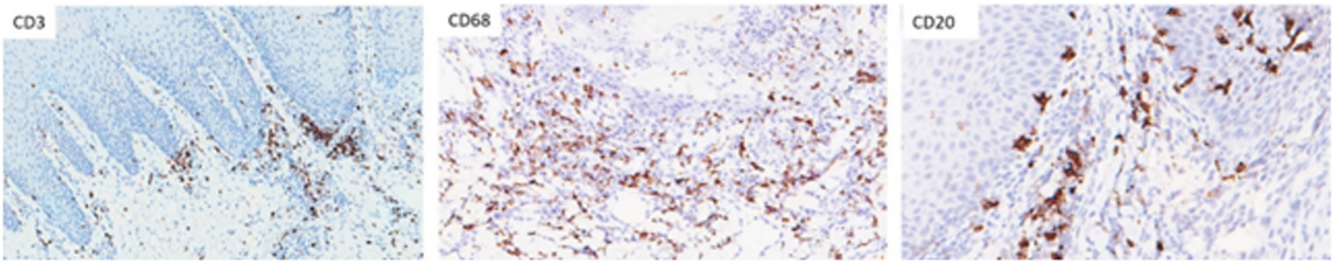

FIGURE 6 Immunohistochemical characterizations of the inflammatory infiltrate consisting of CD3-positive T-lymphocytes (on the left – Immunohistochemistry anti-CD3, 10×), CD20-positive B-lymphocytes (in the middle – Immunohistochemistry anti-CD20, 40×), and CD68-positive monocytes/macrophages (on the right – Immunohistochemistry anti-CD68, 20×).

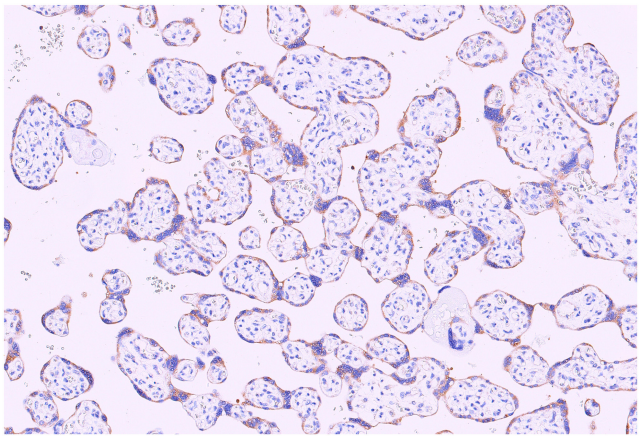

FIGURE 7 The placenta of a COVID-19-positive patient used as immunohistochemical positive control (immunohistochemistry anti-SARS-CoV-2, 20×).

Several studies have been published in the literature reporting on several late signs/symptoms frankly attributable, according to the authors, to manifestations of the post-COVID condition, including lung fibrosis, venous thromboembolism (VTE), arterial thromboses, cardiac thrombosis and inflammation, stroke, "brain fog," dermatological complications, and overall mood dysfunctions. Also, many studies clearly state that the impact of COVID-related long-time signs/symptoms affecting the general population should not be never underestimated as potentially could occur in diverse organs and systems (lungs, brain, cardiovascular system, kidneys, and the neuromuscular system or more than one synchronously) (Bussani et al., 2023; Mannucci et al., 2022; Mantovani et al., 2022) Additionally, it surely represents as a major challenge as PCS surely manifests differently by sex, age, the severity of symptoms of the acute COVID-19 phase, host healthy status, and already suffered medical conditions.

To date, as regards the acute phase of infection, the occurrence of different types of oral lesions vary from diffuse/multifocal erythema to vesiculobullous lesions or ulcers with certain diagnoses achieved by conventional histological and immunohistochemical analysis too (Favia et al., 2021; Soares et al., 2021, 2022). From a pathogenic point of view, such oral lesions are mainly caused by thrombotic vascular occlusion of small and medium size vascular structures, mainly with total occlusion in small vessels and partial in larger. (Favia et al., 2021) As for the oral involvement in PCS, to date, only one case series reported the occurrence of oral lesions in patients with post-COVID conditions (Rafałowicz et al., 2022) but authors limited their report to the clinical aspect only, lacking histopathological investigations; in addition, other papers described the persistence of taste dysfunction far beyond 3 months from the onset of acute illness (Mastrangelo et al., 2021; Srinivasan, 2021). PCS affecting the oral cavity may be variably the result of stress, trauma, and inadequate oral hygiene but more generally of vasculitis, multiorgan disorders, opportunistic infections, reinfection, or dysfunction of the immune system, thus representing the true complexity of the possible long COVID conditions for oral medicine/pathology specialists. Indeed, each case shows a strictly individual course with different general and local symptoms in post-COVID exactly as the acute phase of infection, but additionally with variable occurrence time and persistency too (Bezerra et al., 2022; Gutierrez-Camacho et al., 2022; Melo Neto et al., 2020; Paces et al., 2020; Tomo et al., 2020).

The clinical features of the herein reported PCS oral lesions could easily support their virus-related occurrence as mainly represented by (mostly) wide and (often) irregular ulcers frequently with ulcerative-necrotic-hemorrhagic areas, or by lesions difficult to ascribe to a specific oral mucosa disease already known or a systemic one with oral involvement, so overlapping these COVID-19 related while, microscopically, some difference may be underlined.

As well recognized by Soares et al. (2021, 2022), the main histological aspects of Sars-CoV-2 infection in oral mucosa lesions could be so variably summarized: epithelial vacuolization with occasional exocytosis and a chronic inflammatory infiltrate in the connective tissue mostly composed of lymphocytes and macrophages, rarely of polymorphonuclear neutrophils, differently staged thromboses of small arteries and associated with hemorrhagic areas, neoangiogenesis, almost constant granular cytoplasmic positive staining for the spike protein in the epithelial cells (including the vacuolated cells of the superficial layers, thus probably representing a direct viral cytopathic effect) as well inflammatory and endothelial ones; weak-to-moderate angiotensin-converting enzyme 2 (ACE2) (known as a functional receptor for SARS-CoV-2, located on the host cytomembrane binding the spike protein and so facilitating viral penetration virus in the host cell) positivity in the epithelium while strong in endothelial cells.

Such data reported that spike protein-positive cells of the oral epithelium in COVID-19 patients are detectable in all the epithelial layers, including the vacuolated cells of the superficial layers, thus resembling related to direct viral effects (Soares et al., 2021, 2022); also, Marques et al. (2022) recently demonstrated by a cytological study such positivity also in desquamated epithelial cells from the dorsum of the tongue of 10 out of 14 COVID-19-positive patients and in absence of oral lesions.

In the PCS oral lesions we studied, the histological investigations showed that the epithelial cells of the basal and spinosum layers showed a weak SARS-CoV-2 positivity progressively decreasing or absent in the uppers as well as from the lesion towards the periphery until completely disappearing, thus supporting the Sars-CoV-2-related aetiopathogenesis of the oral mucosa lesions herein described. Additionally, the inflammatory infiltrate was mainly composed of lymphocytes in COVID-19-related oral lesions, which are strongly reduced (almost absent) in post-COVID-19 related ones and replaced by CD68- and CD163-positive cells, including phagocytic neutrophils, macrophages, dendritic cells, natural killer cells, and mast cells; such immune cells potentially form an integrated defensive system against pathogens or induce adaptive mucosal immunity, as widely discussed in the literature for several years now (Gutierrez-Camacho et al., 2022; Holmgren & Czerkinsky, 2005; Yuan & Walker, 2004).

Overall, our data provide consistent evidence that SARS-CoV-2 can potentially persist in the oral mucosa after the acute phase and can be responsible for lesions too. As for other organs/systems, it is unclear whether oral mucosa provides a survival advantage to the virus (as for many other viruses) or a reinfection (without adjunctive signs/symptoms) has occurred or it represents (as part of the gastrointestinal tract) a reservoir of viruses like the salivary glands. Yet, probably, the oral epithelial cells sustain a persistent SARS-CoV-2 infection eliciting a continuous immune response, so behaving like the nasal epithelial cells, as recently demonstrated in vitro by Gamage et al. (2022); in fact, authors identify three aspects of the host–pathogen interaction which likely contribute to the prolonged SARS-CoV-2 replication, such as the virus capability to infect host cells while evading a tissue antiviral response, a limited cell death within the infected epithelium itself, basal cells proliferation for replacing these lost during infection. Effectively, in our histological samples too, we constantly found SARS-CoV-2 (although weak) positiveness of the basal and spinosum layers while missing in the upper layers. Those findings, similar to the nasal epithelium, are coherent with an active inflammatory and immune response to the viral presence/persistency and also, further support the explanation of the negative RT-PCR test as superficially performed.

Recently, a similar persistent infection responsible for the virus-related disease has been further demonstrated in the lung by Bussani et al. (2023), reporting on patients recovered from COVID-19 (and remaining negative for the following 9 months) but died from lung pathologies resembling the acute phase ones, showing in the post-mortem analysis positivity for SARS-CoV-2 antigens in the bronchial cartilage chondrocytes, para-bronchial gland epithelial cells, vascular pericytes, and endothelial cells, whereas negativity of the respiratory epithelium, so concluding that virus may persist in specific cells of the lungs also with a PCR test negative.

As for the immunohistochemical findings in our patients, it hard to assess with certainty whether the virus was still vital or not at the moment of the observation. However, we used a kind of antibody that only recognizes intact N-protein, suggesting a low probability of finding immunopositivity on tissues with the postinfection degraded virus. Moreover, a recent study reported that N-protein is detectable in saliva some days before symptom onset and that N-protein levels correlate with C_t values for viral RNA in saliva, being rapidly degraded after the resolution of symptoms (Shan et al., 2021). However, the immunohistochemical positivity for N-protein is related to the virus presence and its role in the etiopathogenesis of the oral lesions, independently from the vitality of the virus at the moment of observation. If the virus was not involved in the oral lesions' development, probably we would not have observed such immunopositivity for N-protein, which indicates that the virus has been present at some moment in the lesions, even though it may have not been vital at the time of the observation. For these reasons, we believe that our observations, in any case, demonstrate the etiopathogenetic link between the virus and the oral lesion onset.

Understanding the mechanism of pathogenesis of long COVID could provide additional information on it, but to date, it has been assessed by several studies only that SARS-CoV-2 is capable of persisting months after infection in several tissue reservoirs apart from the respiratory tract, renal and cardiac system, gastrointestinal tract, muscular tissues, brain and lymph nodes, and salivary glands, thus providing a persistent systemic immune imbalance (Mehandru & Merad, 2022).

For all the aforementioned reasons and as reported by Bezerra et al. (2022) too, a long-term follow-up by routine intraoral examination should be set in previously diagnosed COVID-19 patients for early detection of oral manifestations too, as the oral counterpart in the complex spectrum of PCS condition as nowadays recognized (Higgins et al., 2021; Sudre et al., 2021; WHO, 2021). Further studies are surely needed to better define the true etiopathogenic mechanism of oral mucosa lesions occurring in PCS patients, overall considering the recent identification both of Sars-Cov-2 and PCS, overall complexity, the broad spectrum of clinical manifestations in the whole organism and especially in the oral cavity, particularly aimed to fully understand if the oral cavity may represent a target organ also in PCS and the mechanism of interaction of SARS-CoV-2 with the mucosal immune system, and, also, underlining possible benefits of the mucosal immune system activation by oral or intranasal vaccines nowadays under experimentation (Mudgal et al., 2020).

AUTHOR CONTRIBUTIONS

Luisa Limongelli: Writing – original draft; writing – review and editing; project administration. **Gianfranco Favia:** Writing – original draft; writing – review and editing; project administration. **Eugenio Maiorano:** Formal analysis; methodology;

validation. **Antonio D'Amati:** Methodology; validation; formal analysis. **Alberto Pispero:** Investigation; methodology; data curation. **Giuseppe Ingravallo:** Methodology; validation; formal analysis. **Giuseppe Barile:** Investigation; methodology; data curation. **Angela Tempesta:** Writing – review and editing; data curation. **Fabio Dell'Olio:** Writing – review and editing; data curation. **Rosaria Arianna Siciliani:** Investigation; writing – review and editing. **Saverio Capodiferro:** Project administration; supervision; conceptualization; writing – original draft; writing – review and editing.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

PATIENT CONSENT STATEMENT

Patients released informed consent both for diagnostic and therapeutic procedures and for the possible use of biological samples for research purposes.

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