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SARS-CoV-2 endothelial infection causes COVID-19 chilblains: histopathological, immunohistochemical and ultrastructural study of 7 paediatric cases

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Running head: Coronavirus in COVID toes

What's already known about this topic?

- Despite the high number of cases of chilblains seen during the COVID-19 pandemic, a definite causative role for SARS-CoV-2 has not been proven yet.
- Different pathogenetic hypotheses, including coagulation anomalies, interferon release, and external factors have been proposed.

What does this study add?

- The demonstration of SARS-CoV-2 in endothelial cells of skin biopsies by immunohistochemistry and electron microscopy confirms these lesions are part of the spectrum of COVID-19.
- Virus-induced vascular damage and secondary ischemia could explain the pathophysiology of COVID-19 chilblains.
- Our findings support the hypothesis that widespread endothelial infection by SARS-CoV-2 could have a pathogenetic role in the severe forms of COVID-19.

SUMMARY

Background: Chilblains ("COVID toes") are being seen with increasing frequency in children and young adults during the COVID-19 pandemic. Detailed histopathological descriptions of COVID-19 chilblains have not been reported, and causality of SARS-CoV-2 has not been established yet.

Objective: To describe histopathological features of Covid-19 chilblains and explore the presence of SARS-CoV-2 in the tissue.

Methods: We examined skin biopsies from 7 paediatric patients presenting with chilblains during the COVID-19 pandemic. Immunohistochemistry for SARS-CoV-2 was performed in all cases and electron microscopy in one.

Results: Histopathology showed variable degrees of lymphocytic vasculitis ranging from endothelial swelling and endothelialitis to fibrinoid necrosis and thrombosis. Purpura, superficial and deep perivascular lymphocytic inflammation with perieccrine accentuation, oedema, and mild vacuolar interface damage were also seen. SARS-CoV-2 immunohistochemistry was positive in endothelial cells and epithelial cells of eccrine glands. Coronavirus particles were found in the cytoplasm of endothelial cells on electron microscopy.

Conclusions: Although the clinical and histopathological features were similar to other forms of chilblains, the presence of viral particles in the endothelium and the histological evidence of vascular damage, support a causal relation of the lesions with SARS-CoV-2. Endothelial damage induced by the virus could be the key mechanism in the pathogenesis of COVID-19 chilblains and perhaps also in a group of patients severely affected by COVID-19 presenting with features of microangiopathic damage.

INTRODUCTION

Acral purpuric lesions identical to chilblains are being seen with an exceedingly high frequency in children and young adults during the coronavirus disease-2019 (COVID-19) pandemic worldwide.¹⁻³ They have been subject of numerous mentions in the non-specialized media usually under the designation "COVID toes".⁴ Most patients have been negative for severe acute respiratory syndrome-coronavirus-2 (SARS-CoV-2) when tested by PCR of nasopharyngeal and oropharyngeal swabs, and less than 50% have a history of exposure to positive household contacts or previous history of mild upper respiratory or gastrointestinal symptoms.¹

Despite the high number of cases, a definite causative role for SARS-CoV-2 has not been proven. Several pathogenetic hypotheses, including coagulation anomalies, interferon release, and external factors have been proposed.^{5,6} On the other hand, the histopathological features of Covid-19 related chilblains and the presence SARS-CoV-2 in the lesions have not been addressed. We present the histopathological, immunohistochemical and ultrastructural features of 7 children with chilblains presenting shortly after the COVID-19 outbreak peak in Madrid, Spain, and propose a causative role for SARS-CoV-2.

PATIENTS AND METHODS

Retrospective case series of paediatric patients diagnosed clinically and histopathologically with chilblains during the COVID-19 pandemic. Approval from the institutional Ethics Committee and Board was obtained. Standard informed consents were obtained for recording images and for skin biopsies in all patients.

The patients were seen in the Emergency Department and then referred to the Dermatology Department of a children's hospital in a period of 4 weeks (April-May 2020), shortly after the peak of incidence of COVID-19 in Madrid. Six of them (cases 1 to 6) were included in a previous report focused on clinical and epidemiological features of 22 patients with similar lesions.¹

Seven biopsy specimens from 7 patients with skin lesions clinically diagnosed as COVID-19 related chilblains were examined. All the biopsies were taken from lesions located on feet or toes.

Clinical parameters were recorded including age, sex, personal history of previous diseases, exposure to potentially infected household contacts, skin symptoms, location of lesions, systemic symptoms, therapies administered and follow-up. Laboratory analyses performed were also recorded, as well as SARS-CoV-2 PCR from nasopharyngeal and oropharyngeal swabs.

All the biopsies were assessed on multiple serial sections stained with hematoxylin & eosin (H&E), Mowry's colloid iron and periodic acid–Schiff (PAS). The following histological features were recorded: vacuolar changes of the basal layer, exocytosis of lymphocytes, necrotic keratinocytes, erosion/ulceration of the epidermis, parakeratosis, spongiosis, oedema of the superficial dermis, perivascular superficial inflammatory infiltrate, perivascular deep inflammatory infiltrate, perieccrine lymphoid cell infiltrate, lymphocytic lobular panniculitis, fibrinoid material within the vessel walls, thrombi in papillary dermal vessels, thrombi in reticular dermis/subcutis vessels, lymphocytic infiltration of dermal vessels, purpura, dermal mucin, and basal membrane thickening. In each case, these features were scored as absent (-), mild/focal (+), moderate (++) or marked/extensive (+++).

Immunohistochemical stains with antibodies against CD3, CD4, CD8, CD20, CD30 and CD61 (Dako, Glostrup, Denmark) were performed in all the samples.

For SARS-CoV / SARS-CoV-2 immunostains the tissue was cut from paraffin blocks in 3 µm sections using a microtome and stretched on a water bath at 40°C, mounted on glass slides and incubated at 60°C for 1 hour. The slides were deparaffinized in xylene and hydrated in ethanol. Antigen retrieval was performed with 10 mM citrate buffer (pH 6.0) in a PT Link (Agilent Technologies, Santa Clara, CA, USA) at 95°C for 20 min. We used a monoclonal antibody (1A9, dilution 1:200, GeneTex Inc., Irvine, CA, USA) against the spike protein of SARS-CoV / SARS-CoV-2. The staining was visualized with 3-Amino-9-ethylcarbazole substrate-chromogenic (Envision system-HRP/AEC, Dako, K4004, Santa Clara, CA, USA) for 4 minutes, counterstained with Mayer's hematoxylin, and mounted with commercially ready to use Dako Ultramount Aqueous Permanent Mounting Medium. The staining was carried out with a Dako Autostainer Link 48 Link (Agilent Technologies, Santa Clara, CA, USA). Negative controls (tonsils from non-COVID-19 patients) were included together with each sample. The antibody was previously optimized using sections of COVID-19 lungs from autopsies as positive controls and different inflammatory skin conditions as negative controls.

We evaluated the relative proportion of T and B cells, the proportion of helper to cytotoxic T cells and the presence of activated lymphocytes. The presence and location of the virus was recorded as present (+) or absent (-). CD61 was done to highlight the presence (+) or absence (-) of intravascular platelet thrombi.

Transmission electron microscopy (EM) examination was performed in case 2.

All the morphologic, histochemical, and immunohistochemical parameters were assessed by 2 of the authors (IC & AT) and SARS-CoV / SARS-CoV-2 stained slides were seen by 4 (IC, AT, LR & CS).

RESULTS

Clinical features

Our 7 patients (4 male, 3 female; 11-17 years old) had skin lesions on the toes and lateral aspects of their feet and heels consistent with chilblains (Figure 1). They were minimally painful or pruritic. The hands were also affected in one patient who had associated lesions of erythema multiforme on the elbows and knees.⁷ Of note, no patient had a history of rheumatic disease, lupus erythematosus, Raynaud's phenomenon, acrocyanosis or previous chilblains, and 2 of them were on treatment with methylphenidate hydrochloride for more than 1 year at the same dose for attention deficit hyperactivity disorder (ADHD). The lesions had been present 4 to 30 days before the biopsy procedure. In all patients, the lesions had a benign outcome, with no significant systemic complaints and gradual spontaneous resolution was achieved in all of them after 8 weeks of follow up (Table 1).

SARS-CoV-2 PCR from nasopharyngeal and oropharyngeal swab was negative in all cases tested (6/6). PCR tests were performed between 1 and 21 days (median 10) from the beginning of the skin lesions. Coagulation studies were normal in 6 patients tested. D-dimer levels in serum were measured in 6 cases and were minimally elevated in one case (900 ng/ml; normal < 500 ng/ml), but this abnormal result had no clinical significance, with the patient showing good health, no systemic symptoms and other coagulation tests within normal limits. Full blood count was normal in all tested cases.

Histopathology

The biopsies showed similar findings with variable intensity (Table 2). All showed a mild interface dermatitis featuring vacuolar degeneration of the basal epidermal layer. Exocytosis of lymphocytes was seen in 3 cases and scattered necrotic keratinocytes in 4. Lymphocytic vasculitis was demonstrated in all biopsies. Lymphocytes infiltrated the wall of dermal venules and arterioles. Endothelialitis, defined as swollen endothelial cells separated from the underlying basement membrane by subendothelial lymphocytes, was frequently observed

(Figure 2). Indirect features of vascular damage such as red cell extravasation and dermal oedema were present in all cases. Fibrinoid necrosis of vessels was seen in 2 biopsies and microthrombosis in 4. These thrombi were noted in papillary dermal capillaries as well as involving reticular dermis vessels (Figure 3). Transmural lymphocytic infiltration of a large subcutaneous vessel, not associated with fibrinoid necrosis or thrombosis, was noted in one case.

A superficial and deep angiocentric and eccrinotropic lymphocytic infiltrate was seen in all samples. Inflammation extended to the subcutaneous fat in the 6 biopsies where subcutis was represented and involving mostly the fat lobule. The inflammatory infiltrate was predominantly composed of small lymphocytes. Large activated lymphocytes were noted only in 2 cases (cases 2 and 3) with more severe inflammation. Plasma cells were only focally seen in these 2 biopsies showing more dense inflammation.

Immunohistochemistry (IHC)

The inflammatory infiltrate was predominantly composed of mature T cells (CD3+) with a predominance of helper T lymphocytes (CD4+) over cytotoxic T lymphocytes (CD8+). Only scattered mature B lymphocytes (CD20+) were seen except for case 5, where aggregates of B cells were noted in the centre of dense nodular lymphoid nodules. Scattered CD30+ cells were observed in cases 2 and 3. CD61 highlighted the presence of small microthrombi in 4 cases, including ones where thrombi were not apparent on H&E stained sections (case 6).

Cytoplasmic granular positivity for SARS-CoV-2 spike protein was mainly demonstrated in endothelial cells of the capillary and post-capillary venules of the upper dermis and also in epithelial cells of the secretory portion of eccrine units in all cases (Figure 4).

Electron microscopy

Ultrastructural examination revealed the presence of round membrane-bound structures within the cytoplasm of endothelial cells showing an electro-lucent centre, and surrounded by tiny spikes, giving them a halo-like appearance. Their mean diameter was 92.26 nm (80.76-109.76 nm), and the mean thickness of the spikes was 13.18 nm (12.36-13.88 nm). Based on previous descriptions in the literature, these structures were interpreted as coronavirus-like particles⁸⁻¹⁴ (Figure 4). Tubulo-reticular inclusions (TRI) were also found within the endothelial cells, similarly to other descriptions of SARS-CoV-2 and SARS-CoV infections^{8,15}.

DISCUSSION

Lymphocytic vascular damage was the hallmark feature in biopsies from our 7 patients with COVID-19 related chilblains. The intensity of the vasculitic damage ranged from minor findings, such as endothelial cell tumefaction and mild lymphocytic infiltration of the walls, to more severe signs like fibrinoid necrosis and thrombosis. The spectrum of histopathological features did not correlate with the duration of the lesions.

Vasculitis is defined as inflammation directed at vessels, which compromises or destroys the vessel wall leading to haemorrhagic and ischemic events. The spectrum of vascular reaction to injury is variable.¹⁶ Moderate to severe lymphocytic inflammation of vessels walls was noted in half of our cases, and inflammation was mild in the rest. In all of them, even in those with mild inflammatory infiltrate, indirect evidence of vascular damage and leakage was present in the form of extravasation of erythrocytes and dermal oedema.

The overall clinical and histopathological features in our cases are entirely in keeping with chilblains. Biopsies of chilblains show a moderate to dense superficial and deep perivascular lymphocytic infiltrate with exocytosis to the epidermis and acrosyringia and perieccrine accentuation. A few necrotic keratinocytes and mild vacuolar degeneration of the basal layer can be observed occasionally. Pronounced papillary dermal oedema and spongiosis are often present. Lymphocytic vasculitis is a common feature, and superficial thrombosis is present in some cases. Chilblains may be classified as primary (idiopathic, cold-related) or secondary to underlying conditions. Some authors consider that primary and secondary chilblains cannot be reliably differentiated based only on microscopic features.¹⁷ Perieccrine inflammation and marked dermal oedema have been described as features favouring idiopathic chilblains over chilblains associated with connective tissue diseases, particularly lupus erythematosus, which is favoured by more prominent changes of interface dermatitis.^{18,19} Immunohistochemistry for lymphoid markers is not useful in the differential diagnosis of primary and secondary chilblains, as both show a predominance of mature T cells with only a minor proportion of B cells and some histiocytes. Increased numbers of large CD30+ cells have been described in some cases of idiopathic chilblains with no clinical significance.^{20,21}

Secondary causes of chilblains include connective tissue diseases (lupus erythematosus, Behçet

disease, antiphospholipid syndrome, rheumatoid arthritis, Sjögren syndrome); cryopathies; haemoproliferative or neoplastic diseases; blood hyperviscosity states; genetic diseases (familial chilblain lupus, STING-associated vasculopathy of infantile onset (SAVI), Aicardi-Goutières syndrome, and IRAK4 deficiency); and anorexia or other disorders causing weight reduction.²²⁻²⁴ None of these causes of secondary chilblains was present in our patients. Moreover, cold exposure was unlikely to be the trigger in our cases, since all lesions appeared during the mild spring climate in Madrid.

A causal relationship with COVID-19 has been considered doubtful in this outbreak of cases of chilblains. However, we are not surprised by the high rate of negative PCR tests in our cases considering the reported low rate of positive PCR tests in children with suggestive symptoms of COVID-19.²⁵ We have demonstrated the presence of viral particles within endothelial cells in lesional skin biopsies from patients presenting with chilblains during the COVID-19 pandemic. The positive immunohistochemistry was confirmed by the presence of viral particles in one case using transmission electron microscopy. Our results strongly support a pathogenetic role for SARS-CoV-2 in chilblains presenting during the pandemic. COVID-19 should be included in the list of secondary causes of chilblains, and we think these lesions can be denominated from now onwards as COVID-19 chilblains.

It has been proposed that SARS-CoV-2 uses angiotensin converting enzyme 2 (ACE2) expressed by pneumocytes in the epithelial alveolar lining to infect the host cells and cause lung injury.^{26,27} It is likely that SARS-CoV-2 uses ACE2 to enter vessels, as ACE2 is widely expressed by endothelial cells.^{28,29} ACE2 is also present in epithelial cells of eccrine glands, explaining the positive immunolocalisation of SARS-CoV-2 we have observed in these cells.²⁹

Vascular injury and thrombosis could explain the clinical features of chilblains and related acro-ischemic lesions seen in some patients with COVID-19. Different mechanisms of vascular damage in this setting have been proposed. Varga et al⁸ suggested that recruitment of immune cells, either by direct viral infection of the endothelium or immune-mediated, can result in widespread endothelial dysfunction associated with apoptosis. Magro et al³⁰ demonstrated a pattern of tissue damage consistent with complement-mediated microvascular injury in the lung and skin of five individuals with severe COVID-19. Their findings provide a foundation for further exploration

of the pathophysiologic importance of complement in COVID-19 and could suggest targets for specific intervention.

Histological features of COVID-19 related chilblains have been only scarcely described.^{5,31} Kolivras et al⁵ reported a SARS-CoV-2 PCR positive 23-year-old man with mild systemic symptoms and skin lesions suggestive of chilblains in his feet and toes. A skin biopsy showed features consistent with chilblains, and the authors suggested a possible role for interferon-1 (IFN-1), based on the similarities with chilblain lupus erythematosus. The presence of SARS-CoV-2 particles in endothelial cells in our cases argues against this hypothesis, suggesting SARS-CoV-2 as a direct cause of the endothelial damage and thrombosis instead. A primary thrombotic mechanism appears unlikely for COVID-19 chilblains based on the limited presentation and normal coagulations tests.

Chilblains seen in young people may, to some extent, resemble the severe acral ischemia caused by thrombosis in severely ill COVID-19 patients. These patients are usually elderly, admitted to intensive care units, and show coagulopathy with very elevated D-dimer levels.³² A severity spectrum cannot be ruled out since widespread endothelial infection could contribute to the systemic multi-organ manifestations seen in patients with severe COVID-19. In fact, SARS-CoV-2 has been shown to infect endothelial cells in the skin and other organs in a few cases.^{8,30,33}

Our study has some potential limitations. We acknowledge that the number of patients is small; furthermore, evidence of SARS-CoV-2 infection by PCR and serology could not be obtained. Also, the use of immunohistochemistry for SARS-CoV/SARS-CoV-2 is still restricted and larger studies are necessary to assess with accuracy the role of immunohistochemistry in the diagnosis of COVID-19. Finally, we only carried out electron microscopy in one case; even though the virus was detected, more cases are needed to further support our conclusions.

In conclusion, the presence of SARS-CoV-2 in the endothelium of dermal vessels in skin biopsies of children and adolescents with acute chilblains confirms that these lesions are a manifestation of COVID-19. Their clinical and histopathological features are similar to those of chilblains of other aetiologies, and virus-induced vascular damage could explain their pathophysiology. Our findings support the hypothesis that widespread endothelial infection by SARS-CoV-2 could have a role in the pathogenesis of severe forms of the disease. More studies are needed to understand the reasons why previously healthy children, adolescents and young adults present

with limited skin forms of COVID-19, in contrast with the severe multi-organ presentations seen in older patients with background diseases.

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Figure legends

Figure 1. Cases 2 (A,B) and 6 (C,D). Clinical spectrum of pernioptic acral ischemic lesions.

Figure 2. Case 6, skin biopsy. A) Acral skin showing superficial and deep perivascular inflammation extending into the subcutis and papillary dermal edema (H&E, 20X). B) Mild exocytosis and vacuolar degeneration (H&E, 200X). C-F) Lymphocytic infiltration of vessel walls. Note the lifting of endothelium with underlying lymphocytes in E (arrow) and transmural inflammation of a large subcutaneous vessel in F (H&E, C & E 400X; D & F 200X).

Figure 3. Case 2, skin biopsy. A) Acral skin showing superficial and deep perivascular inflammation extending into the subcutis (H&E, 20X). B) Mild papillary dermal edema, vacuolar degeneration of the basal layer and lymphocytic exocytosis together with prominent red cell extravasation (H&E, 100X). C) Thrombi in superficial dermis vessels (H&E, 200X). D-F) Lymphocytic vasculitis with thrombosis and fibrin deposition in vessel walls (H&E, D 200X; E-F 400X).

Figure 4. A-C. Cases 4-6. Cytoplasmic granular positivity for SARS-CoV-2 spike protein in endothelial cells (IHC, 400X). D. Case 2. Ultrastructural image of an endothelial cell showing coronavirus-like particles consistent with SARS-CoV-2 (arrow), next to a mitochondrion for size comparison (EM, 60000x).

ABBREVIATION LIST

COVID-19: coronavirus disease-19

SARS-CoV-2: severe acute respiratory syndrome-coronavirus-2

H&E: hematoxylin & eosin

PAS: periodic acid–Schiff

EM: electron microscopy

ADHD: attention deficit hyperactivity disorder

IHC: immunohistochemistry

SAVI: STING-associated vasculopathy of infantile onset

STING: stimulator of interferon genes

IRAK4: interleukin-1 receptor-associated kinase 4

ACE2: angiotensin converter enzyme 2

Table 1. Clinical features in 7 children and adolescents with COVID-19 chilblains

Case	Age	Sex	ADHD	Suspected household contact	Respiratory/ GI symptoms	Time to biopsy	Site of lesions	Local symptoms	PCR
1	11 y	F	No	Yes	Yes/Yes	4 days	Feet	-	-
2	13 y	F	Yes	No	Yes/No	11 days	Feet	Mild pain	-
3	15 y	F	No	No	Yes/No	30 days	Feet	-	NA
4	15 y	M	No	Yes	No/No	15 days	Feet	-	-
5	14 y	M	No	No	Yes/No	20 days	Feet	Pruritus	-
6	17 y	M	No	Yes	Yes/No	20 days	Feet, hands	Pruritus	-
7	15 y	M	Yes	Yes	No/No	7 days	Feet	Pruritus	-

ADHD: attention deficit hyperactivity disorder; M: male; F: female. NA: not available

Table 2. Histopathological features of 7 skin biopsies of COVID-19 chilblains in children and adolescents

	Case 1	Case 2	Case 3	Case 4	Case 5	Case 6	Case 7
Basal vacuolar changes	+	++	++	+	+	+	+
Exocytosis	-	++	-	+	-	+	-
Necrotic keratinocytes	+	+	+	-	-	+	-
Erosion/ulceration	-	-	-	-	-	-	-
Parakeratosis	-	+	-	++	++	-	+
Spongiosis	+	++	+	+	+	++	+
Dermal edema	+	++	++	++	+	++	+
Perivascular superficial inflammation	+	+++	+++	+	+	+	+
Perivascular deep inflammation	+	+++	+++	+	+	++	+
Perieccrine inflammation	+	+++	+++	+	+	++	NA
Lymphocytic panniculitis	+	+++	+++	+	+	+	NA
Fibrinoid necrosis	-	+	+	-	-	-	-
Thrombi superficial dermis	-	+	-	-	-	-	-

Thrombi deep dermis	-	+	+	+	+	-	-
Lymphocytic infiltration of vessels	+	+++	+++	+	+	++	+
Purpura	+	+++	+	+	+	++	+
Dermal mucin	-	+	++	+	+	+	-
Basal membrane thickening	-	-	-	-	-	-	-
Other findings	Vascular ectasia	-	-	Vascular ectasia	-	Subcutaneous lymphocytic vasculitis	Vascular ectasia

NA: not assessable

Table 3. Summary of immunohistochemical findings

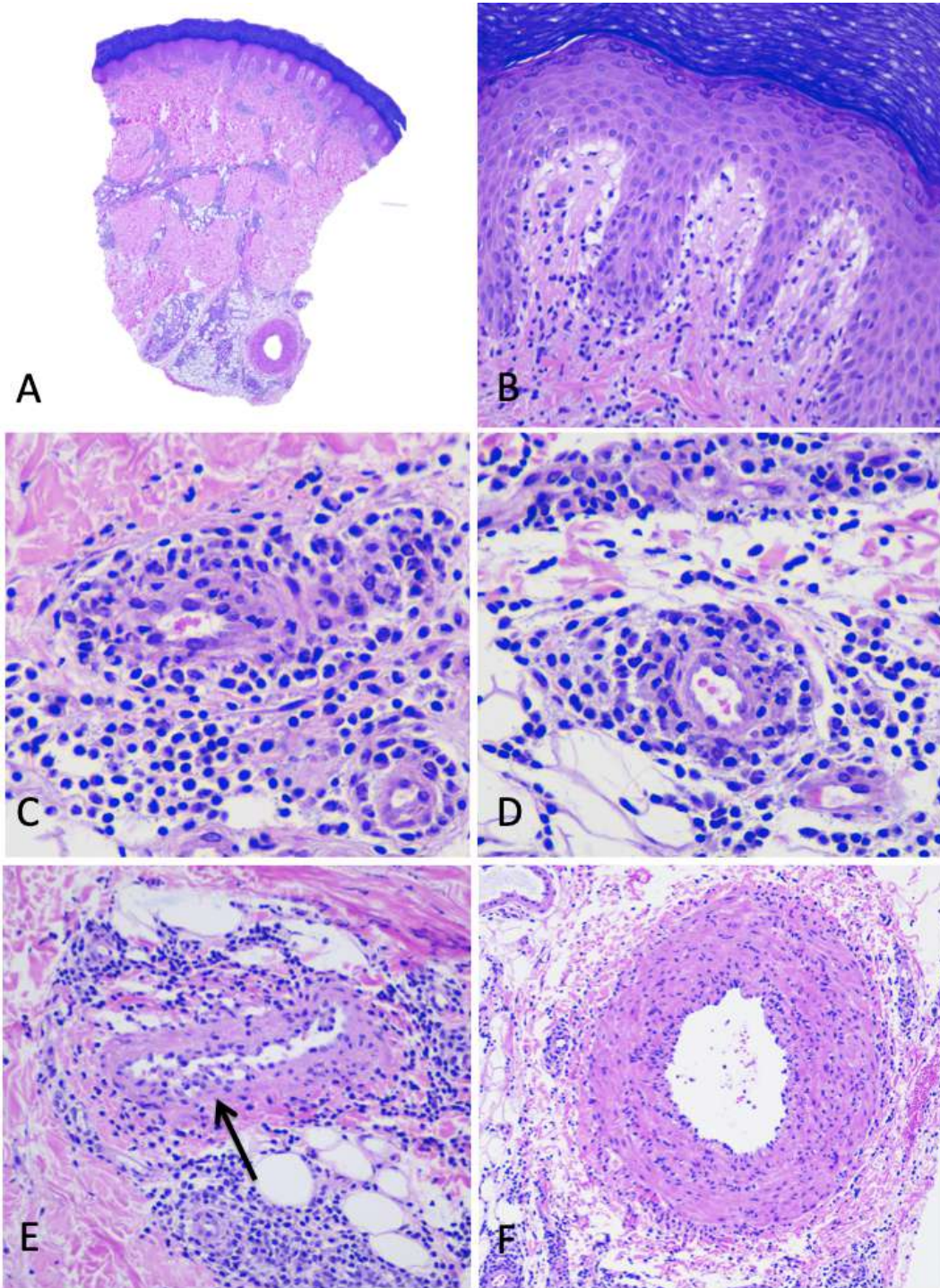
Case	Inflammatory cell phenotype	CD4:CD8 ratio	Thrombi on CD61	SARS-CoV-2 spike protein
1	Mainly CD3+, scattered CD20+	4:1	-	+
2	Mainly CD3+, scattered CD20+ and CD79+, scattered CD30+	1:1	+	+
3	Mainly CD3+, aggregates of CD20+ and CD79a+, scattered	2:1	-	+

	CD30+			
4	Mainly CD3+, scattered CD20+ and CD79a+	2:1	+	+
5	Mainly CD3+, scattered CD20+ and CD79a+	3:1	+	+
6	Mainly CD3+, scattered CD20+ and CD79a	3:1	+	+
7	Mainly CD3+, scattered CD20+	2:1	-	+

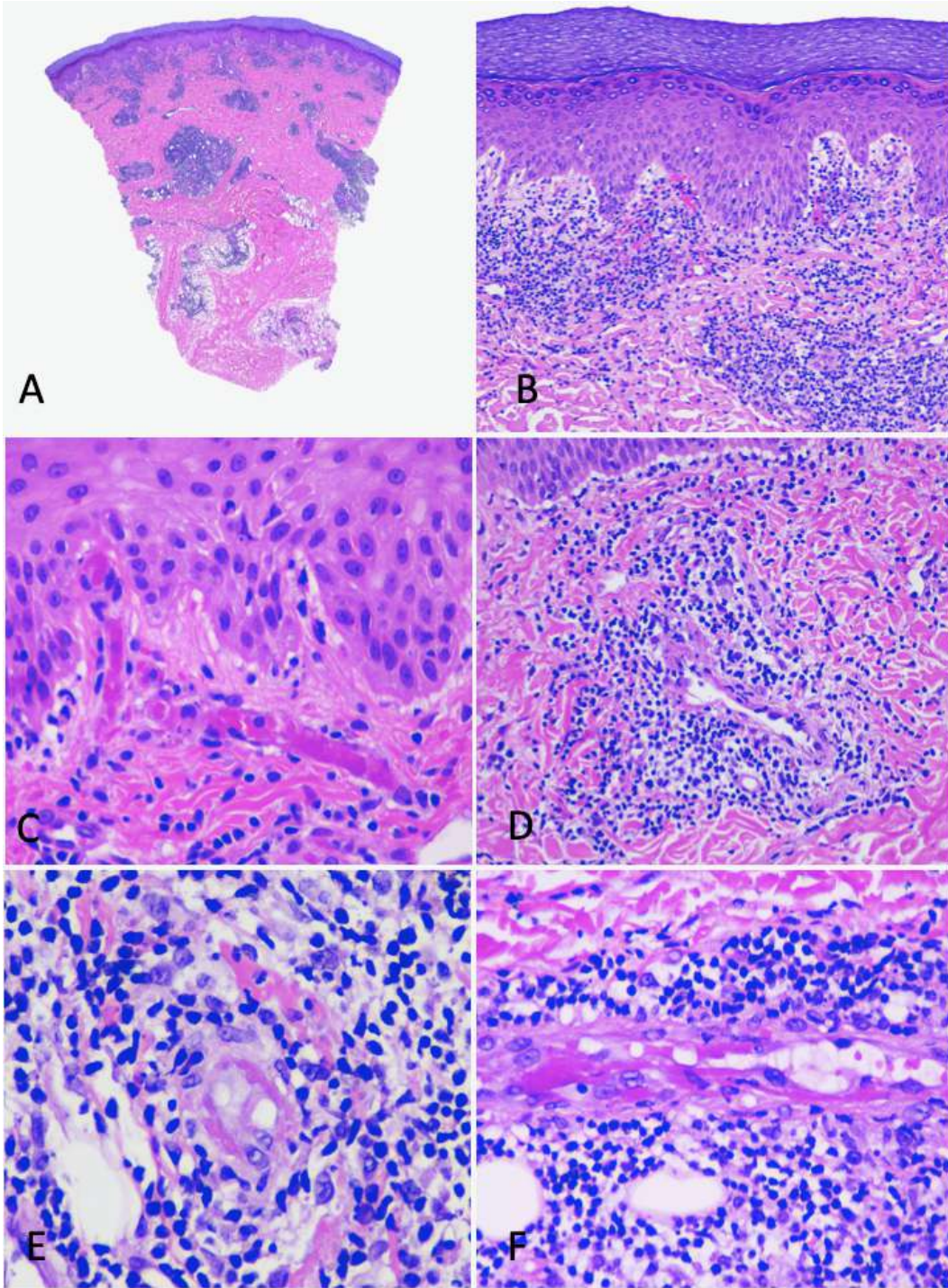
NA: not available



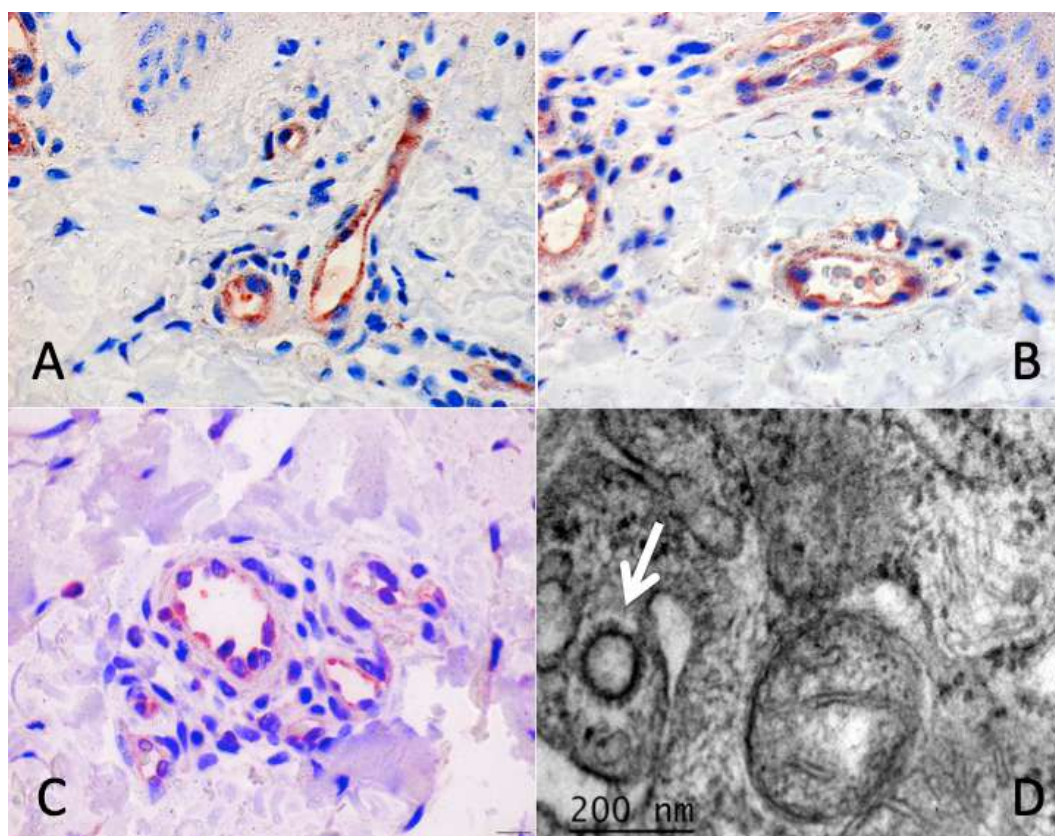
bjd_19327_f1.tiff



bjd_19327_f2.tiff



bjd_19327_f3.tiff



bjd_19327_f4.tiff