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Chilblains observed during the COVID-19 pandemic cannot be distinguished from classic, cold-related chilblains

Background: Type 1 interferon (IFN-I) response induced by SARS-CoV-2 has been hypothesized to explain the association between chilblain lesions (CL) and SARS-CoV-2 infection. *Objective:* To explore direct cytopathogenicity of SARS-CoV-2 in CL and to focus on IFN-I expression in patients with chilblains. *Materials & Methods:* A monocentric cohort of 43 patients presenting with CL from April 2020 to May 2021 were included. During this period, all CL were, a priori, considered to be SARS-CoV-2-related. RT-qPCR on nasopharyngeal swabs and measurements of anti-SARS-CoV-2 antibodies were performed. Anti-SARS-CoV-2 immunostainings as well as SARS-CoV-2 RT-qPCR were performed on biopsy specimens of CL and controls. Expression of MX1 and IRF7 was analysed on patients' biopsy specimens and/or PBMC and compared with controls and/or chilblains observed before the pandemic. Serum IFN-α was also measured. *Results:* RT-qPCR was negative in all patients and serological tests were positive in 11 patients. Immunostaining targeting viral proteins confirmed the lack of specificity. SARS-CoV-2 RNA remained undetected in all CL specimens. MX1 immunostaining was positive in CL and in pre-pandemic chilblains compared to controls. MX1 and IRF7 expression was significantly increased in CL specimens but not in PBMC. Serum IFN-α was undetected in CL patients. *Conclusion:* CL observed during the pandemic do not appear to be directly related to SARS-CoV-2 infection, either based on viral cytopathogenicity or high IFN-I response induced by the virus.

Key words: Chilblains, COVID toes, COVID-19, immunostaining, SARS-CoV-2, Type 1 interferon

A s Coronavirus disease 2019 (COVID-19) peaked in Western Countries in March-April 2020 (first wave) and September-December 2020 (second wave), several cutaneous manifestations associated with SARS-CoV-2 infection were reported, with chilblain lesions (CL) being most frequently observed [1]. The chronological correlation between CL and pandemic peaks of SARS-CoV-2 infections raised concerns in the medical community, particularly in southern countries in which these types of lesions, classically caused by cold exposure, are less common. An association between CL and SARS-CoV-2 infection, as well as their pathophysiology remains debated [2]. RT-qPCR on nasopharyngeal swabs and anti-SARS-CoV-2 antibodies are negative in most patient series reported [3, 4]. Attempts have been made to identify the virus in the lesions, and the role of virus-induced type I interferon (IFN-I) response has been suggested [5-11]. However, the results remain insufficiently validated and many questions remain unanswered [12].

A strong IFN-I response is suggested to be associated with early control of SARS-CoV-2 infection, which could contribute to a symptomless expression of the disease in patients with chilblains. IFN-I is believed to be responsible for clearance of the virus and for muting viral replication without intervention of the adaptive B-cell immune system, which could explain low viraemia, negative RT-qPCR and the absence of SARS-CoV-2 antibodies in CL patients. Accordingly, studies have demonstrated the presence of autoantibodies against IFN-I or inborn errors of immunity in patients with life-threatening COVID-19 [13, 14]. Therefore, a similar mechanism to that of interferonopathies, of which chilblains are known to be one of the clinical manifestations, has been put forward [7, 12].

Supported by positive immunohistochemistry (IHC) with antibodies directed against different parts of the virus, other authors have hypothesized that CL result from endothelial damage and thrombosis caused directly by SARS-CoV-2 skin infection [5, 15, 16]. However, the lack of specificity of anti-SARS-CoV/SARS-CoV-2 immunostaining on skin biopsies has been pointed out and should be interpreted with caution [17, 18].

[a] These authors contributed equally

To further study the pathophysiology of CL observed during the pandemic, the objectives of this study were to: (i) test the reliability of immunostainings for virus identification in skin samples and explore direct cytopathogenicity of SARS-CoV-2 in these lesions; and (ii) focus on IFN-I expression in skin and blood of patients with chilblains.

Patients and methods

Patients

Between April 10, 2020, and May 31, 2021, a total of 43 patients who presented to the Department of Dermatology at Cliniques universitaires Saint-Luc, Brussels, Belgium, with chilblain lesions located on the toes and/or fingers, were included. Twenty-two of these patients were part of our previously reported first study series but were included in the present study for additional analyses that were not performed nor published previously [19].

Twenty healthy control subjects with no skin lesions were enrolled; eight for peripheral blood mononuclear cells (PBMCs) or serum analyses and 12 for skin biopsies, which were collected before 2019. Serum from 29 non-CL patients who suffered from mild to severe forms of SARS-CoV-2 infection were used for the IFNα ELISA. This study and data collection were conducted with the approval of the hospital and faculty institutional review board (Commission d'Ethique Biomédicale Hospitalo-Facultaire) of Université catholique de Louvain, Belgium. Informed consent for all diagnostic procedures was obtained from all study participants or their legal representatives.

Methods

Nasopharyngeal swabs for RT-qPCR analysis to detect SARS-CoV-2 RNA were performed on 42 patients (one patient refused) when they presented with chilblains. In all patients, blood analyses included the following: C-reactive protein, complete blood count, liver and renal functions, prothrombin time and activated partial thromboplastin time, D-dimer levels, fibrinogen, protein electrophoresis, cryoglobulins, tests for antinuclear antibodies and parvovirus B19 serology. Serological testing using three different laboratory methods to detect anti-SARS-CoV-2 Ig (immunoglobulin) M, IgG and/or IgA antibodies was performed in all patients. Interferon regulatory factor 7 (IRF7) and antiviral myxovirus resistance protein 1 (MX1) expression in peripheral blood mononuclear cells (PBMCs) was evaluated in 21 patients and eight healthy controls. Detection of IFNα2 was performed in serum of 22 patients, eight healthy controls, and 29 non-CL patients who suffered from mild to severe forms of COVID-19. Skin biopsy specimens from 37 patients were used for immunohistochemical analyses, including: anti-spike and anti-nucleocapsid antibodies (SARS-CoV-2), anti-angiotensin-converting enzyme 2 (ACE2) and anti-MX1 antibodies. Fourteen skin biopsy specimens from patients with a diagnosis of chilblains before the pandemic (from 2015 to 2019) were used for comparison. IRF7, MX1 and SARS-CoV-2 spike expression were evaluated in skin specimens of seven CL patients and 12 controls.

Detailed methods for SARS-CoV-2 RT-qPCR on nasopharyngeal swabs, SARS-CoV-2, IRF7 and MX1 RT-qPCR on skin, serology testing, skin biopsy procedures, immunochemistry analyses, isolation of PBMCs and stimulation and detection of IFNα2 in serum are provided in the *supplementary material.*

Results

All demographic data are summarized in *supplementary table 1.*

Patients' medical history, demographic, and epidemiologic data

Among the 43 patients included in this study, 11 were children or adolescents (<18 years old), 25 were female and the mean age was 29.52 years (range: 13-61 years). The mean Body Mass Index (BMI) was 21.85 (range: 15.60-38.12) and 14 patients had a BMI < 20.

CL affected exclusively the extremities of the feet in 29 patients and/or hands in 14 patients, and in most cases, were peri-ungual. CL presented as erythematous or purplish-erythematous macules, sometimes with vesicular or bullous lesions at the centre, and in some cases with necrotic areas. Patients complained of pain, burning and/or itching. These characteristic features are suggestive of chilblains (*figure 1A*). No other cutaneous lesions were observed elsewhere.

The mean delay between onset of CL and consultation was 25.15 days (range: 3-180 days). However, this was potentially lengthened by five patients with a delay >60 days (Patients 35, 38, 39, 40, 42, 43) (see *supplementary table 1*). Two of the three patients with the longest delay before consultation (range: 90-180 days) were diagnosed with lupus-chilblains. Eight patients (19%) suffered from CL during both the first wave (March-April, 2020) and the second wave (September-December, 2020) of SARS CoV-2 infections in Belgium. Eight patients (19%) noticed yearly seasonal chilblains, nine (21%) suffered previously from Raynaud's syndrome, 20 (46%) were known to have acryocyanosis, and 18 (41.8%) did not present any history of Raynaud's syndrome, chilblains nor acrocyanosis. Five patients (11%) had a history of documented SARS-CoV-2 infection (positive RT-qPCR from nasopharyngeal swabs) 1.5 to 7 months before CL onset. Four patients had had close contact with a person with confirmed SARS-CoV-2 infection.

Biological analyses

No significant biochemical, autoimmune, haematological, or hemostatic abnormalities were found on blood tests, except for one patient who also suffered from acute myeloid leukaemia. Autoimmune diseases, such as systemic lupus erythematosus and other infections, such as parvovirus B19, were ruled out in all patients. Positive antinuclear antibodies were detected in 11 patients (1/640 titre maximum). After anti-extractable nuclear antigen (ENA) screening, one patient was positive for anti-Ro/anti–Sjögren's-
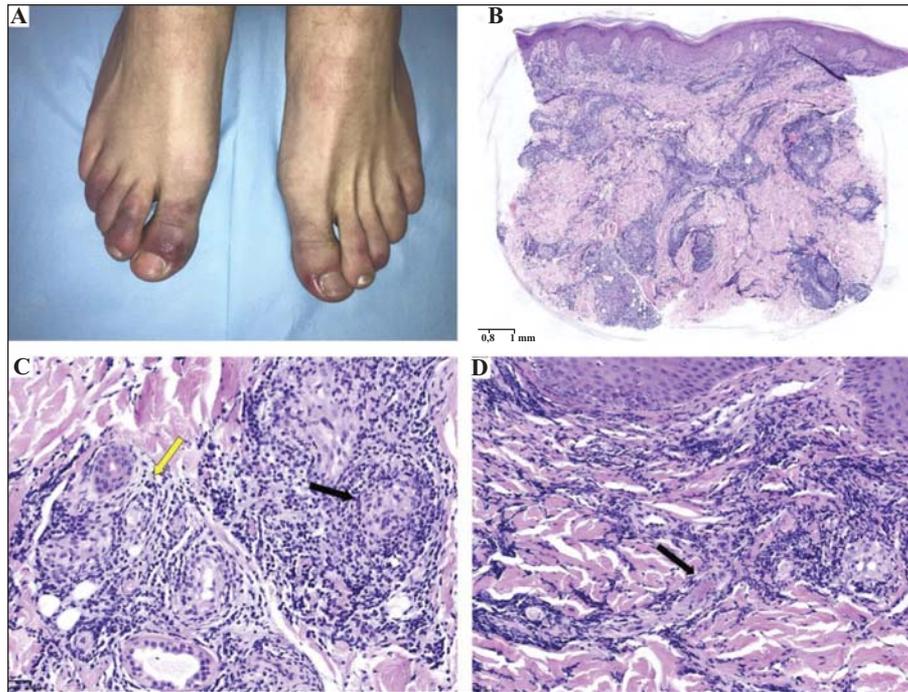
Figure 1. Patient 26. **A**) Clinical presentation of chilblain lesions observed during the pandemic with purplish-erythematous macules located on the toes. **B-D**) Histopathological features showing a very dense and deep inflammatory lymphohistiocytic infiltrate in the dermis (**B**), essentially peri-eccrine (**C**) (yellow arrow) and perivascular with a lymphocytic vasculitis (**C**) (black arrow) as well as microthrombosis (**D**) (black arrow) (haematoxylin and eosin; original magnification: [**B**] x3, [**C**] x20, [**D**] x20).

syndrome-related antigen A (SSA) and another for anti-U1-ribonucleoprotein (U1RNP) antibodies.

RT-qPCR on nasopharyngeal swabs and serological tests for anti-SARS-CoV-2 IgM and IgG

At the time of consultation, SARS-CoV-2 remained undetected by RT-PCR on nasopharyngeal swabs in all tested patients.

Based on the three different methods used, titres for IgM, IgG and IgA antibodies against SARS-CoV-2 were negative in 32 patients and positive in 11 patients (seven with serology Method B and four additional patients with the in-house ELISA serology method) including five with previously documented SARS-CoV-2 infection before the appearance of chilblains (history of positive RT-qPCR on nasopharyngeal swab).

Histological analysis of skin biopsies of CL

Histopathological analysis of skin biopsy samples revealed patterns consistent with typical chilblain in 34 patients with, in some cases, lymphocytic vasculitis and microthrombotic phenomena (*figure 1B-D*); for Patients 38, 41 and 43, the differential diagnosis of lupus-chilblains was considered.

SARS-CoV-2 identification in skin samples and control tissues

Immunohistochemistry for SARS-CoV-2 spike protein showed diffuse non-specific staining in all tissue specimens (*supplementary figure 1*). Immunostaining for SARS-CoV-2 nucleocapsid protein was negative in healthy control skin, in eccrine glands, and in endothelial cells in CL during and before the pandemic (*supplementary figure 2A-C*). Specimens from healthy control lung tissue as well as SARS-CoV-2-infected and non-SARS-CoV-2-infected lung tissues were also negative (*supplementary figure 2D-F*). Cytoplasmic granular positivity was observed in syncytiotrophoblastic cells of the placenta from a SARS-CoV-2-positive patient, whereas endothelial cells remained negative (*supplementary figure 2G*). Healthy at-term placenta from before the pandemic was negative (*supplementary figure 2H*).

Surprisingly, healthy control skin stained positive for ACE2 protein compared to chilblain samples obtained during and before the pandemic which remained negative despite repeated laboratory procedures (*supplementary figure 3A-C*). Immunohistochemical analyses of ACE2 showed scarce expression of ACE2 in uninfected control lungs whereas staining was slightly more significant in SARS-CoV-2-infected lungs and lungs from patients with ARDS before the pandemic (*supplementary figure 3D, E, F*). A similar observation was made between control placenta and SARS-CoV-2-positive placenta (*supplementary figure 3G, H*). RT-qPCR failed to detect SARS-CoV-2 in all CL biopsy specimens.

Interferon signature

Expression of MX1 and IRF7 in unstimulated PBMCs did not differ between healthy controls and CL patients. Stimulation of PBMCs with phytohemagglutinin (PHA) and interleukin (IL)-2 did not significantly increase MX1 and IRF7 expression in healthy controls and CL patients (*figure 2A-D*). Conversely, MX1 and IRF7 expression was significantly increased in skin biopsies from CL patients compared with healthy controls (*figure 2E, F*). MX1 was also detected by immunostaining in CL but similarly in pre-pandemic chilblains, compared to healthy control skin (*figure 3A-C*).

Serum IFNα2 concentration remained undetected in healthy controls and CL patients and was only detected in three out of 29 non-CL patients with SARS-CoV-2 infection (*figure 2G*). These three patients suffered from a severe form of the disease and their serum was collected between three and seven days after symptom onset. IFNα2 was not detected in the supernatant of cultured PBMCs from healthy controls and CL patients (data not shown).

Discussion

RT-qPCR on nasopharyngeal swabs to detect SARS-CoV-2 was negative in all patients tested at the time of CL, and SARS-CoV-2 serology was negative in 32 out of 43 patients (with a rate of positive serology similar to that of the general population at the time) [20]. Recent studies have shown evidence of long-lasting humoral and cellular immunity against SARS-CoV-2 even in mild SARS-CoV-2 infected patients [21]. Other authors point out that a robust innate response to SARS-CoV-2 indeed may explain less severe infection, but does not affect the quality of SARS-CoV-2 specific antibody responses [22]. These observations contradict the hypothesis of an intense interferon response with blocking of antibody production.

Our study confirms the insufficient reliability of immunostaining for SARS-CoV-2 in the skin. SARS-CoV-2 anti-spike immunostaining demonstrated over-sensitivity. SARS-CoV-2 nucleocapsid immunostaining showed opposing results depending on the primary antibody used [17]. Our study confirms that the sole use of immunohistochemistry is currently not recommended for virus detection [18]. SARS-CoV-2 RT-qPCR is probably more reliable for the identification of virus in CL. The functional receptor ACE2 is expressed in many tissues, therefore, it represents a possible route of infection for SARS-CoV [23]. Surprisingly, none of the chilblains (during and before the pandemic) stained positive for ACE2, contrary to healthy control skin, which was notably positive. Yet, inflammatory conditions are expected to influence (increase as well as decrease) ACE2 expression [24, 25]. Therefore, it was not possible to conclude whether the virus is present in CL based on immunostaining.

An association between CL and virus-induced local or systemic IFN-I response could not be demonstrated in our study. IFN-I could not be detected in PBMCs and serum of CL patients, which is not surprising because the IFN produced after acute viral infection is quickly consumed due to ubiquitous expression of its receptor. The occurrence of chilblains, on average more than 20 to 30 days after reported acute SARS-CoV-2 infection or high-risk contact, could therefore explain the absence of IFN-I detection [26]. However, if IFN-I overexpression was the cause of CL, we would have expected to find higher systemic levels of IFN-I in these patients at the time of chilblains as well as other cutaneous or extracutaneous symptoms, as observed in other interferonopathies [27, 28]. Our study did not confirm the results obtained by Hubiche *et al*. who observed increased levels of IFNα after >CD3/R848 stimulation of whole blood [8]. The use of R848, a Toll-like receptor 7/8 (TLR 7/8) agonist, in their protocol may explain this difference. Indeed, R848 induces activation of pDCs, which are strong producers of IFN-I.

The similar overexpression of both MX1 and IRF7 in skin biopsies of pandemic and pre-pandemic chilblains confirms that they are, by definition, inflammatory reactions involving predominantly IFN-I. Therefore, the overexpression of IFN-I would be expected in CL of all aetiologies and does not allow to demonstrate a correlation with SARS-CoV-2 infection.

Most of our patients with CL had a similar profile - mainly adolescents and young adults; BMI often below 20; a frequent history of Raynaud's disease, acrocyanosis or even pre-pandemic chilblains; and a significant number (11 patients, 25·5%) with positive antinuclear antibodies. This profile is very similar to that encountered in patients most likely to develop classic cold-induced chilblains [29].

This is in accordance with the hypothesis shared by many other authors that CL are more likely caused by the lockdown-imposed sedentary lifestyle which precipitates chilblains or their relapse in these predisposed patients [19, 30-32]. The association with sedentary lifestyles during quarantine, cold environments, and barefoot exposure on cold floors, together with a pathogenesis similar to that of classic cold-related chilblains, is likely to have largely contributed to the increase of CL observed during both waves of the pandemic.

In order to avoid selection bias, we purposely included every patient presenting with CL during the pandemic, regardless of their pre-existing conditions (*i.e.* history of seasonal chilblains) or final diagnosis (*i.e.* lupus-chilblains). Therefore, our patient sample is representative of the actual population affected by chilblains during this period, as all CL were considered in the same unbiased manner with no *a priori* aetiology assigned.

Finally, it is also surprising that CL have been underreported in some countries also affected by the pandemic [33]. In the case of a direct association with the SARS-CoV-2 virus or with a subsequent IFN-I response, similar reports would be expected from all countries/locations with comparable infection rates and climate conditions [34]. A possible explanation for this could be the difference in containment measures between countries. For example, Nordic countries, for which there were no reports of chilblain outbreaks during this period, did not impose strict confinement, as opposed to southern European countries [33].

To conclude, our data do not allow to differentiate between chilblains observed during the COVID-19 pandemic and classic idiopathic chilblains. We suggest a pathogenesis similar to that of cold-related chilblains, precipitated by a lockdown-imposed sedentary lifestyle, and in particular, related to the presence of predisposing factors.
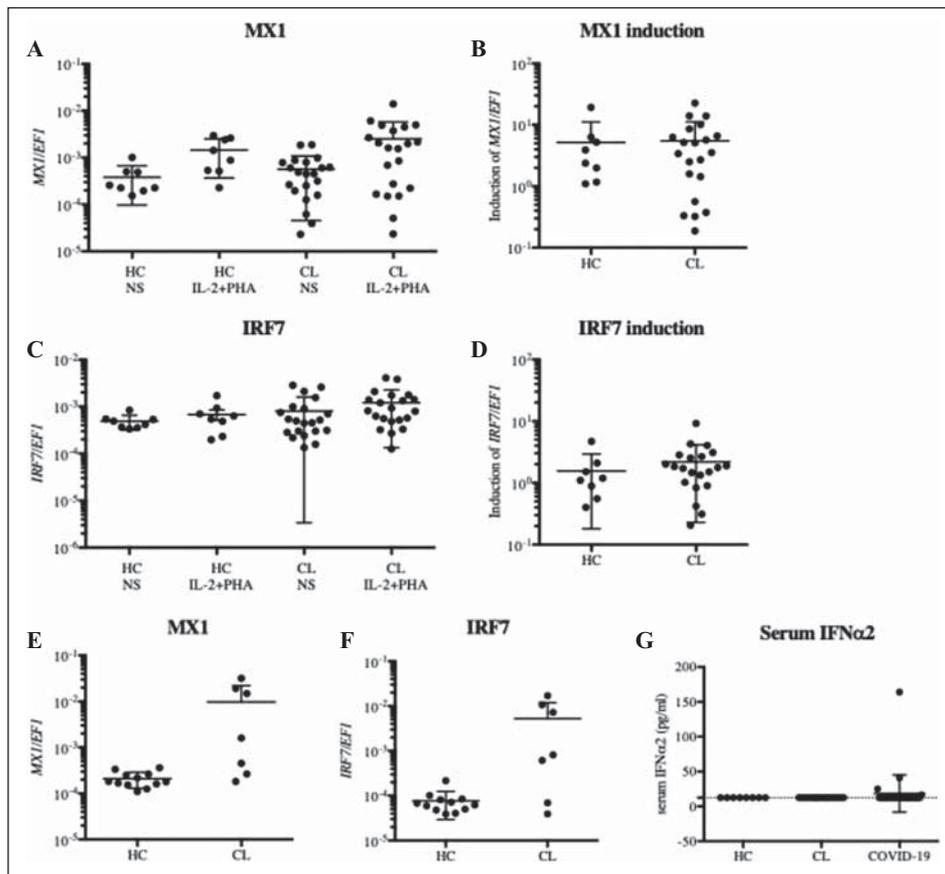
Figure 2. Interferon signature in patients with CL. **A-D**) PBMCs from healthy controls (HC, *n*=8) and CL patients (*n*=21) were cultured without stimulation (NS) or with PHA+IL-2 stimulation for 72 hours. RNA was then isolated and qPCR to determine MX1, IRF7 and EF1 mRNA expression was performed. MX1 and IRF7 expression was normalized against EF1. **B, D**) Induction was calculated by comparing the expression of MX1 and IRF7 in stimulated PBMCs versus unstimulated PBMCs. **E-F**) Skin biopsies were performed on healthy skin of HC (*n*=12) and skin lesions of CL patients (*n*=7). RNA isolation and qPCR to determine MX1, IRF7 and EF1 mRNA expression were performed as described for PBMCs. **G**) IFNα2 concentration in serum of HC (*n*=8), CL patients (*n*=23) and SARS-CoV-2 infected patients (*n*=29) was measured by ELISA. The cut-off for detection of IFNα2 was 12.5 pg/mL. Data are shown as mean ±SD.*$p \leq 0.05$ and **$p \leq 0.01$ ([**A**], [**C**], [**G**]: Kruskal-Wallis test with Dunn's multiple comparisons test; [**B**], [**D**], [**E**], [**F**]: Mann-Whitney test).
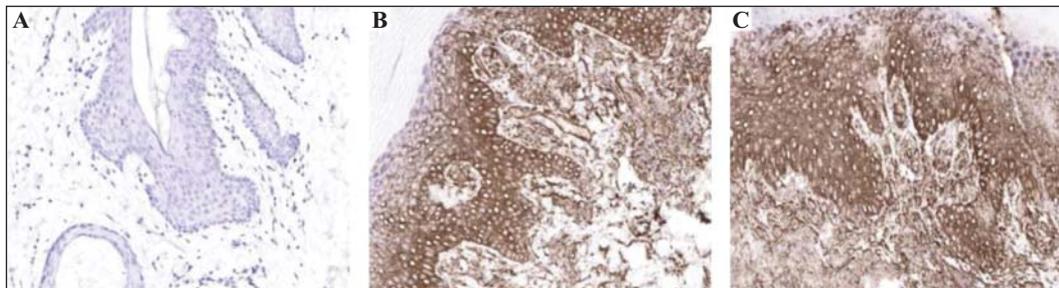

Figure 3. Immunohistochemistry for antiviral myxovirus resistance protein 1 (MX1) showing weak non-specific staining of the basal layer of keratinocytes from healthy skin (**A**), and diffuse positive staining of the epidermis and a lymphohistiocytic infiltrate from a pre-pandemic idiopathic chilblain (**B**) and a chilblain during the pandemic (**C**) (haematoxylin and eosin; magnification: x20 for all images).

Study limitations

We could not provide a contemporary reliable control of non-pandemic chilblains for MX1 and IRF7 RT-qPCR, as well as PBMC and serum IFN expression, since, as we were still in a pandemic period, we could not obtain samples of chilblains that we could say with certainty were not related to SARS-CoV-2 infection. All consenting patients who presented to our department with CL during the pandemic were

included, with no *a priori* aetiology assigned. However, 14 skin biopsy specimens from patients with a diagnosis of chilblains before the pandemic (from 2015 to 2019) were retrieved for immunostaining comparisons. ■

Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1684/ejd.2022.4260.
Table S1. Demographic data of 43 patients with chilblain-like lesions.
Figure S1. Immunohistochemistry for SARS-CoV-2 spike protein showing: diffuse weak non-specific staining of keratinocytes and sebaceous glands of healthy skin (**A**); diffuse non-specific staining of eccrine glands and endothelial cells as well as a lymphocyte infiltrate in a CL (**B**) and a pre-pandemic idiopathic chilblain (**C**); diffuse non-specific staining of bronchial epithelium, pneumocytes and endothelial cells of healthy lung (**D**); non-specific staining of pneumocytes, endothelial cells and lymphocytes of SARS-CoV-2-positive lung (**E**) and pre-pandemic ARDS lung (**F**); and diffuse staining of synciotiotrophoblasts and endothelial cells of healthy at-term pre-pandemic placenta (**G**) and placenta of a SARS-CoV-2-positive patient (**H**) (haematoxylin and eosin; magnification: x20 for all images).
Figure S2. Immunohistochemistry for SARS-CoV-2 nucleocapsid protein showing negative staining of healthy skin (note the scarce, non-specific, positive staining of the basal layer of the epidermis) (**A**); negative staining of a CL (**B**) and a pre-pandemic idiopathic chilblain (**C**); negative staining of healthy lung (**D**), COVID-19 lung (**E**) and pre-pandemic ARDS lung (**F**); and negative staining of healthy at-term pre-pandemic placenta (**G**). **H**) Strong and clear granular positivity of syncitiotrophoblasts of the placenta of a SARS-CoV-2 positive patient, with respect to endothelial cells (haematoxylin and eosin; magnification: x20 for all images).
Figure S3. Immunohistochemistry for angiotensin-converting enzyme 2. **A**) Positive staining of eccrine glands in healthy skin. Eccrine glands and the endothelium of a CL (**B**) and a pre-pandemic idiopathic chilblain (**C**) remain strictly negative, and some rare pneumocytes in healthy lung stain positive (**D**), however, staining is slightly stronger in pneumocytes, endothelial cells and lymphocytes of SARS-CoV-2-infected lung (**E**) and pre-pandemic ARDS lung (**F**). **G**) Positive staining of syncitiotrophoblasts of healthy at-term pre-pandemic placenta which is stronger in the placenta of a SARS-CoV-2-positive patient (**H**) (haematoxylin and eosin; magnification: x20 for all images).

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