SARS-CoV-2 Detection by Digital Polymerase Chain Reaction and Immunohistochemistry in Skin Biopsies from 52 Patients with Different COVID-19-Associated Cutaneous Phenotypes

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SARS-CoV-2 detection by digital polymerase chain reaction and immunohistochemistry in skin biopsies from a cohort of 52 patients with COVID-19-associated cutaneous manifestations

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ABSTRACT

Background: COronaVIrus Disease 19 (COVID-19) is associated with a wide spectrum of skin manifestations, including urticarial rash, erythematous maculopapular rash, papulovesicular exanthema, chilblain-like acral lesions, livedo reticularis-livedo racemosa-like pattern and purpuric “vasculitic” pattern. The presence of SARS-CoV-2 RNA in the skin of patients with COVID-19-associated cutaneous manifestations has been demonstrated only in a limited number of isolated case reports.

Objective: To demonstrate the persistence of SARS-CoV-2 RNA in skin samples from patients with different COVID-19-related cutaneous clinical phenotypes.

Methods: Demographic and clinical data from a large cohort of patients (n=52) with COVID-19-associated cutaneous manifestations from five Dermatology Units of the Lombardy region (Italy) were collected. Immunohistochemistry and digital PCR (dPCR) were performed in all skin samples. RNA-FISH was used to confirm the presence SARS-CoV-2 RNA in samples with positivity of either ddPCR or immunohistochemistry.

Results: Ten out of 52 patients (19%) tested positive for spike protein in immunohistochemistry, five of whom had also positive testing in ddPCR. Of the latter, one tested positive both for RNA in situ hybridization (ISH) and ACE-2 in immunohistochemistry while another one tested positive for nucleocapsid protein. Among the five patients positive in ddPCR, two cases presented with chilblain-like acral lesions, two cases with erythematous maculopapular rash and one with urticarial rash. Eleven out of 52 patients showed positivity only for nucleocapsid protein in immunohistochemistry. Among the twelve patients positive for nucleocapsid, one case presented with chilblain-like acral lesions, five cases with confluent erythematous/maculopapular/morbilliform rash, three cases with papulovesicular exanthem and three with purpuric “vasculitic” pattern.

Conclusions: In our study, SARS-CoV-2 was detected only in 38% (20/52) of skin samples, without any apparent associations between SARS-CoV-2 skin positivity and specific cutaneous phenotypes, suggesting that the pathophysiology of the skin lesions mostly depends on the activation of the immune system against the virus. The combination of spike and nucleocapsid immunohistochemistry have higher diagnostic yield than ddPCR. Peripheral skin persistence of SARS-CoV-2 may depend on timing of skin lesions, viral load and effectiveness of the immune response against the virus.

INTRODUCTION

Dermatologists of Lombardy, the first region struck by the COronaVIrus Disease 19 (COVID-19) due to the severe acute respiratory virus 2 (SARS-CoV-2), were among the first ones to have the opportunity to examine skin lesions of infected patients [1,2]. Six main clinical phenotypes of COVID-19-associated cutaneous manifestations—*i.e.*, urticarial rash, confluent erythematous-maculopapular-morbilliform rash, papulovesicular exanthema, chilblain-like acral pattern, livedo reticularis-livedo racemosa-like pattern and purpuric "vasculitic" pattern—have been initially described [3]. In an Italian multicenter study, the two most common presentations were confluent erythematous-maculopapular-morbilliform rash and chilblain-like acral pattern, which accounted for 25.7% and 24.6% of the 187 patients included in the statistical analysis, respectively. [4]

The pathophysiology underlying these skin manifestations and, in particular, the role of SARS-CoV-2 in triggering the different clinical phenotypes remain elusive. Moreover, data about the presence of the virus in skin samples are controversial [5-18].

Indeed, a number of studies, mainly carried out on patients with chilblain-like lesions, failed to confirm the presence of SARS-CoV-2 in the skin. [5-11] In contrast, isolated reports of patients with different COVID-19-related cutaneous manifestations (maculopapular eruptions [13], leukocytoclastic vasculitis [14], urticarial vasculitis [15], lupus tumidus-like lesions [16] and chilblain-like lesions [17,18] in whom SARS-CoV-2 was detected in the skin by different methods—*i.e.*, PCR [13, 14, 19] immunohistochemistry [15,17-19, 20] or FISH [16]—have emerged from literature.

In order to clear up this still undefined topic demonstrating the possible presence of SARS-CoV-2 in skin samples of patients with COVID-19-related cutaneous manifestations, we collected samples, clinical and laboratory data from a large cohort of patients from five different Dermatology Units of the Lombardy region.

METHODS

Patients

A total of 52 patients with COVID-19-associated cutaneous manifestations who were examined between March 1, 2020 and May 30, 2020 were included in the study. Each participating center was asked to provide data on the basis of the following patient inclusion criteria: (1) an age of 18 years or older, (2) probable or laboratory-confirmed COVID-19, and (3) the presence of COVID-19–related skin manifestations confirmed by an expert dermatologist. A COVID-19 diagnosis was considered to be laboratory confirmed in the case of a nasopharyngeal swab with a positive result for SARS-CoV-2 RNA or positive serology result for anti–SARS-CoV-2 IgG/IgM antibodies. COVID-19 was considered probable in any patient meeting the clinical criteria (dry cough, fever, dyspnea, the sudden onset of hyposmia or hypogeusia) who had been in close contact with someone with confirmed COVID-19 in the 14 days before symptom onset. A history of new medications in the 15 days before the onset of the skin manifestations was considered an exclusion criterion. The data included sex, age at the time of onset of COVID-19, the presence/absence of comorbidities, cutaneous patterns, the presence/absence of mucous lesions, the duration of skin manifestations, skin-related symptoms, systemic symptoms, the duration of systemic symptoms, the latency between the cutaneous manifestations and systemic symptoms, death, and the severity of COVID-19.

Clinical assessment

Systemic symptoms were taken from the charts of hospitalized patients or reported by outpatients and assessed by a physician (a pulmonologist or a specialist in internal/emergency medicine or infectious diseases). The duration of the skin manifestations was directly evaluated by a dermatologist in the case of hospitalized patients or reported by outpatients. Each patient was examined at least twice (during the period of skin manifestations and after their resolution). The severity of COVID-19 was classified as asymptomatic, mild (in the presence of fever, cough, and/or gastrointestinal symptoms with no imaging sign of pneumonia), moderate (in the presence of dyspnea and/or radiologic findings of pneumonia), or severe (a need for invasive assisted ventilation, the occurrence of thromboembolic events, or death) [21] and was assessed by considering the worst systemic symptoms over the entire course of the disease, as shown in hospital records or self-reported by outpatients.

Laboratory

SARS-CoV-2 RNA detection and quantification in nasopharyngeal swabs

Our clinical microbiology laboratory utilized the Allplex 2019-nCoV Assay (Seegene) for molecular detection of SARS-CoV-2 in COVID-19 patients. Allplex 2019-nCoV Assay is a multiplex real time PCR (RT-PCR) assay for simultaneous detection of 3 target genes of SARS-CoV-2 (RdRP, N, and E, respectively). A specimen was considered positive if the gene target had a cycle threshold (Ct) of < 40.

SARS-CoV-2 RNA detection and quantification in skin biopsies

In order to identify even minimal quantities of viral RNA in peripheral tissues, a droplet digital PCR (ddPCR) approach was chosen. This technique allows an absolute, precise, and ultrasensitive quantitation of nucleic acids. Briefly, skin biopsy specimens were fixed in 10% buffered formalin, embedded in paraffin, and sectioned into 10-µm sections. Four paraffin-embedded sections were processed for manual RNA extraction with the MagMAX™ FFPE DNA/RNA Ultra Kit (Thermo Scientific), following the manufacturer's instruction. Then, 5.5ul of eluted RNA were retrotranscribed with the One-Step RT-ddPCR Advanced Kit for Probes (BioRad), and SARS-CoV-2 genomic RNA quantified by means of the QX200 Droplet Digital PCR System (ddPCR, BioRad) using SARS-CoV-2 Droplet Digital PCR Kit (BioRad, CA, USA), which has recently obtained the emergency use authorization from the American FDA. SARS-CoV-2 quantification was expressed in copy number/Δl of reaction.

Immunohistochemical analysis

Immunohistochemical analysis was performed using SARS/SARS-CoV-2 Coronavirus Nucleocapsid Antibody (Monoclonal antibody, B46F) [MA1-7404] at a dilution of 1:100, SARS-CoV-2 Spike Antibody (Polyclonal Rabbit IgG) [GeneTex®, GTX135356] at a dilution of 1:300 and Human/Hamster ACE-2 Antibody (Monoclonal Mouse) [MAB933, R&D Systems, Minneapolis, MN USA]) at a dilution of 1:300

The immunostaining protocols were optimized and validated to avoid nonspecific staining that is commonplace and give confidence in the sensitivity of the protocol and quality of the tissues.

Placenta tissue of five COVID-19 patients and lung tissue of five COVID-19 patients were used as positive controls. To check monoclonal antibody specificity, immunohistochemical analysis on ten skin biopsies (five psoriasis and five basal-cell carcinoma) was performed.

Single-molecule RNA in situ hybridization

All cases were also inspected with RNAscope technology (Advanced Cell Diagnostic, Newark, CA) an RNA in situ hybridization (ISH) technique described previously [22]. Paired double Z oligonucleotide probes were designed for hybridization to the target RNA by using custom software. The RNAscope 2.5 LS Probe V-

nCoV2019-S (catalog number 848568; Advanced Cell Diagnostics, Newarki, CA) was used. The RNAscope 2.5 LSx Reagent Kit-Brown (Advanced Cell Diagnostics) in combination with a BOND-III Automated stainer (Leica Biosystems, Buffalo Gorve, IL) was used to process the samples according to manufacturer's recommendations. The RNA integrity of each sample was evaluated with a probe designed for hybridization specifically to the ubiquitin C and cyclophilin B housekeeping genes. The negative control background staining was evaluated using a probe specific to the bacterial dapB gene. Each punctate dot signal representing a single target RNA molecule could be detected with standard light microscopic analysis.

RNAscope Image acquisition and data analysis

Images were captured using Axio Zeiss Scope A1 microscope. RNA marker was analyzed based on the average RNA dot number per cell. RNA quantity was scored based on manual counting following RNAscope Reference Guide described as follows.

Staining results were categorized into five grades according to the number of dots visualized under the brightfield microscope. 0: no staining or less than 1 dot to every 10 cells (40x magnification); 1+: 1-3 dots/cell (visible at 20-40x magnification); 2+: 4-10 dots/cell, very few dot cluster (visible at 20-40x magnification); 3+ : >10 dots/cell; and more than 10% positive cells have dot clusters (visible at 20x magnification); and 4+: > 10 dots/cell, and more than 10% positive cells have dot clusters (visible at 20x magnification).

Ethical approval and consent to participate

The study was conducted in accordance with the Declaration of Helsinki, and the full protocol was approved by the institutional review board of the ethics committee of the principal investigator's center (Fondazione IRCCS Ca' Granda Ospedale Maggiore Policlinico, Milan, Italy; protocol no. 464_2020). All of the participants enrolled in the study gave their written informed consent.

RESULTS

Clinical features of skin manifestations and COVID-19

The demographic and clinical features of the 52 patients are summarized in Table 1. The patients were predominantly males (n = 30; 58%), and their median age at the time of the diagnosis of COVID-19 was 57 years (IQR, 25). Of the 52 patients, 7 (11%) developed urticarial rash; 19 (36%) confluent erythematous/maculo-papular/morbilliform rash; 12 (23%) papulovesicular exanthem; 4 (8%) a chilblain-like acral pattern; 1 (2%) a livedo reticularis/racemosa-like pattern; and 7 (13%)

a purpuric vasculitic pattern. The median duration of cutaneous manifestations was 13 days [IQR (8-23) 15]. COVID-19 was laboratory confirmed in 38 (73%) patients and was regarded as probable in the remaining 14 (27%). Mean latency time between COVID-19 onset and skin manifestations was 21.5 [IQR (7.5-29) 21.5]. Five patients (10%) were asymptomatic, 20 (38%) had mild disease, 16 (31%) had moderate disease, and 11 (21%) had severe disease.

Table 1. Demographic and clinical data of the patients included in the study

Median age at time of COVID-19 onset, years (IQR)		57 [IQR (44.75-69.75) 25]
Males, n (%)		30 (58%)
Females, n (%)		22 (42%)
Median latency between cutaneous manifestations and systemic symptoms, days (IQR)		21.5 [IQR (7.5-29) 21.5]
Median duration of cutaneous manifestations, days (IQR)		13 [IQR (8-23) 15]
Cutaneous phenotypes	**Urticarial rash, n (%)**	7 (11%)
	Confluent erythematous/maculo-papular/morbilliform rash, n (%)	19 (36%)
	Papulovesicular exanthem, n (%)	12 (23%)
	Chilblain-like acral pattern, n (%)	4 (8%)
	Livedo reticularis/racemosa-like pattern, n (%)	1 (2%)
	Purpuric "vasculitic" pattern, n (%)	7 (13%)
COVID-19 severity	**Asymptomatic, n (%)**	5 (10%)
	Mild, n (%)	20 (38%)
	Moderate, n (%)	16 (31%)
	Severe, n (%)	11 (21%)
Diagnosis of COVID-19	**Suspected, n (%)**	14 (27%)
	Laboratory-confirmed, n (%)	38 (73%)

Droplet digital PCR findings

In five patients, the droplet digital PCR (ddPCR) approach revealed the presence of SARS-CoV-2 RNA in paraffin-embedded formalin-fixed skin specimens, albeit with minimal viral loads. However, since ddPCR quantifies target nucleic acid sequences by directly enumerating many positive partitioned reactions, without the need for a standard curve, and thus allowing a specific, ultrasensitive and absolute quantitation of nucleic acids—detection limit of 0.1copies/ul reaction (extracted RNA)—the results obtained clearly indicated the presence of viral RNA in skin samples. Positive patients presented with three different phenotypes: urticarial rash (n=1); confluent erythematous maculopapular morbilliform rash (n=2) and chilblain-like acral lesions (n=2) and had had either mild (n=3) or severe (n=2) COVID-19. In this group, mean latency time between COVID-19 onset and skin manifestations was 29.8 days, mean duration of skin lesions was 30.4 while mean duration of systemic symptoms was 24.8. Three patients had mild COVID-19 infection while two were severe cases. Nasopharyngeal swab for SARS-CoV-2 was positive in 3 patients, not performed in 1 patient and negative in 1 patient while serology tests for SARS-CoV2 was positive only in 1 patient and not performed in the remaining cases. Clinical data of patients who tested positive in ddPCR are summarized in Table 2.

Immunohistochemical analysis

The immunohistochemical analysis for detection of SARS-CoV-2 nucleocapsid protein revealed positive staining for the endothelium of small dermal vessel in 3 patients, 2 of whom presented with purpuric “vasculitic” pattern and one with chilblain-like acral lesions and positive staining for the dermal eccrine sweat glands has been detected in 8 patients, 5 of whom presented with confluent erythematous/maculopapular/morbilliform rash and 3 with papulovesicular exanthem. A patient with purpuric “vasculitic” pattern showed positive nucleocapsid staining for the dermal sebaceous glands. In this group, mean latency time between COVID-19 onset and skin manifestations was 11 days, mean duration of skin lesions was 23 days while mean duration of systemic symptoms was 33.3. 2 patients had asymptomatic COVID-19 infection, 4 patients had a mild form, 4 had a moderate form and 2 were severe cases. Nasopharyngeal swab for SARS-CoV-2 was positive in 8 patients, not performed in 2 patients and negative in 2 patients while serology tests for SARS-CoV-2 was positive only in 2 patients, not performed in 8 patients and negative in 2 patients.

Immunohistochemical SARS-CoV-2 spike protein staining showed positivity for the endothelium of small dermal vessel in 3 patients and for the endoluminal portion of the dermal eccrine glomeruli in 7 patients. Positive patients presented with four different phenotypes: confluent erythematous maculopapular morbilliform rash (n=5), chilblain-like acral lesions (n=3), urticarial rash (n=1) and papulovesicular exanthem (n=1). In this group, mean latency time between COVID-19 onset and skin manifestations was 22.4, mean duration of skin lesions was 32.5 days while mean duration of systemic symptoms was 16.6. Only 1 patient had asymptomatic COVID-19 infection, 5 patients had a mild form, 2 had a moderate form and 2 were severe cases. Nasopharyngeal swab for SARS-CoV-2 was positive in 6 patients, not performed in 1 patient and negative in 3 patients while serology tests for SARS-CoV-2 was positive only in 1 patient, not performed in 6 patients and negative in 3 patients.

Immunohistochemistry for detection of ACE-2 receptor protein revealed positive staining for the endothelium of small dermal vessel in 2 patients, 1 of whom presented with confluent erythematous maculopapular morbilliform rash and one with chilblain-like acral lesions. In this group, mean latency time between COVID-19 onset and skin manifestations was 34 days, mean duration of skin lesions was 39 while mean duration of systemic symptoms was 26. 1 patient had mild COVID-19 infection while the other one was a severe case. Nasopharyngeal swab for SARS-CoV-2 was positive in 1 patient and not performed in the other one while serology tests for SARS-CoV-2 were not performed in either patient. Clinical data of patients who tested positive in immunohistochemistry are summarized in Table 2.

RNAscope findings

Skin sections were evaluated using RNAscope technology with the V-nCoV2019-S probe for SARS-CoV-2 spike protein mRNA. The RNA ISH assay confirmed SARS-CoV-2 presence in the skin lesions only in one case presented with chilblain-like acral lesions (Table 2). This revealed a positivity of Grade 1+, according to the grading classification scale that was previously described, in the upper layer of parakeratosis (Figure 1)

Table 2

Demographics data			**Detection methods of SARS-CoV-2**					**Clinical data**									
Patient ID	Sex	Age	NUCLEOCAPSID*	SPIKE*	ACE2*	RNA ISH SPIKE	ddPCR	Clinical phenotype	Latency between COVID-19 onset and skin manifestations (days)	Duration of skin lesions (days)	Duration of systemic symptoms	Severity	Systemic symptoms	Others complications	Nasopharyngeal swab positivity for SARS-CoV2	Serology tests for SARS-CoV2	Follow-up
1	M	20	_	"+" small dermal vessels	"+" small dermal vessels	"+" upper layer of parakeratosis	"+"	chilblain-like acral	46	60	7	mild	hypo/ageusia, hypo/anosmia	none	not performed	not performed	CR
2	F	55	_	"+" dermal eccrine sweat glands	"+" small dermal vessels	_	"+"	confluent erythematous/ maculopapular/morbilliform rash	22	18	45	severe	fever,cough,dyspnea,pneumonia	Invasive ventilation (tracheostomy), urinary tract infection	yes	not performed	CR
3	M	57	"+" dermal eccrine sweat glands	"+" dermal eccrine sweat glands	_	_	"+"	confluent erythematous/maculo papular/morbilliform rash	28	8	59	severe	fever, cough, dyspnea, pneumonia, hepatosplenomegaly, thromboembolism	Invasive ventilation (tracheostomy), lung aspergillosis	yes	not performed	CR

4	F	34	_	"+" dermal eccrine sweat glands	_	_	"+"	urticarial rash	24	16	10	mild	hypoageusia	none	yes	yes	CR
5	M	28	_	"+" small dermal vessels	_	_	"+"	chilblain-like acral	29	50	3	mild	nausea,diarrhea,abdominal pain	none	no	not performed	CR
6	M	25	_	"+" small dermal vessels	_	_	_	chilblain-like acral	30	40	7	asymptomatic	Na	none	no	no	CR
7	M	52	_	"+" dermal eccrine sweat glands	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	5	6	9	moderate	fever, dyspnea, nausea, diarrhea, abdominal pain,hypo/anosmia, hypo/ageusia	none	yes	not performed	CR
8	F	44	"+" dermal eccrine sweat glands	"+" dermal eccrine sweat glands	_	_	_	papulovesicular exanthem	5	13	9	mild	fever,cough,abdominal pain,hypo/anosmia, hepatosplenomegalia	none	yes	no	CR
9	M	78	_	"+" dermal eccrine sweat glands	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	15	70	10	mild	fever,hypo/anosmia	none	no	not performed	CR
10	M	40	_	"+" dermal eccrine sweat glands	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	20	44	7	moderate	fever,dyspnea,diarrhea,abdominal pain	none	yes	no	CR
11	F	55	"+" dermal small vessels	_	_	_	_	purpuric “vasculitic” pattern	9	35	10	mild	fever,hepatosplenomegalia	arthritis	no	not performed	CR
12	F	86	"+" dermal eccrine sweat glands	_	_	_	_	papulovesicular exanthem	5	8	30	mild	Cought	neurological complications	yes	yes	resolution with sequele
13	M	40	"+" dermal eccrine sweat glands	_	_	_	_	papulovesicular exanthem	5	40	6	mild	fever,cought	none	not done	not performed	CR
14	M	62	"+" dermal small vessels	_	_	_	_	purpuric "vasculitis" pattern	9	15	50	severe	pharyngodynia,coryza,dyspnea,pneumonia,	none	yes	not performed	CR

15	M	18	"+" dermal small vessels	_	_	_	_	chilblain-like acral	5	20	na	asymptomatic	Na	none	not done	not performed	CR
16	M	77	"+" dermal eccrine sweat glands	_	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	15	60	60	moderate	fever, cough, pneumonia, dyspnea, pharyngodyna, coryza	none	yes	yes	resolution with sequele
17	M	63	"+" dermal sebaceous glands	_	_	_	_	purpuric "vasculitic" pattern	8	20	45	moderate	pneumonia, nausea, diarrhea, abdominal pain	none	yes	not performed	CR
18	F	63	"+" dermal eccrine sweat glands	_	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	21	40	na	asymptomatic	Na	none	no	no	CR
19	M	40	"+" dermal eccrine sweat glands	_	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	15	10	39	moderate	dyspnea,pneumonia	Sepsis from multi-resistant St.Epidermis	yes	not performed	resolution with sequele
20	F	60	"+" dermal eccrine sweat glands	_	_	_	_	confluent erythematous/ maculopapular/morbilliform rash	7	7	25	moderate	fever, cough, pharyngodyna, dyspnea, penumonia	Cognitive decay	yes	not performed	resolution with sequele

DISCUSSION

COVID-19 patients may present with a heterogeneous spectrum of cutaneous manifestations related to SARS-CoV-2 infection but the influence of SARS-CoV-2 in inducing the different cutaneous phenotypes needs to be clarified. In particular, the direct cytopathogenic viral effect ("viral eruption") versus the indirect interaction of the skin with the virus due to the virus-induced activation of the immune system ("paraviral eruption") may act in different cutaneous presentations. It has been hypothesized that the varicella-like papulovesicular eruption represents a classic viral exanthema following active viremia while chilblain-like acral lesions are paraviral in their origin, depending on the activation of the skin immune system in response to SARS-CoV-2 infection [23].

In our study, the presence of SARS-CoV-2 RNA in lesional skin was detected by means of ddPCR in five patients associated to three distinct phenotypes, i.e chilblain-like acral lesions (2 cases), erythematous-maculopapular rash (2 cases) and urticarial rash (1 case). Interestingly, immunohistochemical analysis confirmed the presence of SARS-CoV-2 revealing a positive staining for SARS-CoV-2 spike protein in the small dermal vessel's endothelium and eccrine glands of these cases. Moreover, no association was found between presence of SARS-CoV-2 in the skin and COVID-19 severity or latency time between COVID-19 onset and COVID-19-related cutaneous manifestation appearance. In line with these findings, all the ddPCR-negative cases were negative also on immunohistochemistry for

spike protein, except for five patients who showed positive dermal vessel endothelial staining for spike protein. Thus, it may be postulated that in a small portion of patients with COVID-19-associated cutaneous manifestations, COVID-19 can be diagnosed only through skin molecular analysis due to false negative nasopharyngeal swabs or lack of humoral immunity development leading to negative serology [13]. As proof of this, another patient in our cohort resulted SARS-CoV-2 positive on skin ddPCR and immunohistochemistry but negative on nasopharyngeal swab PCR and serology test.

Cases of COVID-19-related cutaneous manifestations positive for SARS-CoV-2 in lesional skin were reported only anecdotally, making it challenging a precise estimation of skin positivity for SARS-CoV-2 among these patients. In our cohort of COVID-19-associated cutaneous manifestation patients, which is up to now the largest one investigated by means of SARS-CoV-2 skin ddPCR and immunohistochemistry, around 40% of patients (five patients with immunohistochemistry and ddPCR and 15 patients with immunohistochemistry only) tested positive for SARS-CoV-2, suggesting that the virus spreads to the skin only in a minority of COVID-19 patients and in most cases the pathophysiology of the cutaneous manifestations is “paraviral”. Based on the endothelial positivity for SARS-CoV-2 on immunohistochemistry in eight of our cases and in some cases published in the literature [15,17-20], it must be assumed that the viral spreading to the skin occurs through the circulatory stream. However, the viral detection in the skin was not associated with a distinct cutaneous phenotype in our series, making it conceivable that it mainly depends on the viral load and the effectiveness of the immune response – either humoral or cell-mediated – against the virus. However, the role of cytokine-driven inflammation, which plays a crucial part at systemic level leading to the so-called cytokine storm [24], in the pathogenesis of skin lesions needs to be explored. Only two of our seven patients with vasculitic lesions had presence of SARS-CoV-2 in the endothelium. Considering that the virus has been observed at the endothelial level in the skin, one would expect a higher frequency of SARS-CoV-2-positive vasculitic manifestations. Instead, reports of vasculitis with virus presence are only anecdotal, including two cases of urticarial vasculitis and a case of leukocytoclastic vasculitis, in which SARS-CoV-2 was detected by means of immunohistochemistry and PCR, respectively [14,15].

Immunohistochemistry seemed to be the most sensitive method, particularly the detection of nucleocapside antigen, which has shown 100% sensitivity and 100% specificity and is more sensitive than spike protein antibody for detecting early infection [25]. The integration of immunohistochemical staining for nucleocapsid, spike and ACE-2 antigens allowed us to demonstrate the viral presence in the skin in 20 out of 52 patients. Thus, immunohistochemistry appeared more sensitive than ddPCR in our cohort of patients. Moreover, the RNAscope ISH positivity gave us the possibility of a direct visualization of the virus while retaining tissue morphology, a feature that is lost in other methods such as ddPCR.

We speculate that ddPCR could have more sensitively detected the viral particles in the skin if performed at specific time points. Indeed, anti-nucleocapsid and anti-spike antibodies appear between day 8 to day 14 after initial symptoms [25]. The presence of viral particles in the epithelium on ISH confirms the fact that SARS-CoV-2 can be found either in the dermis and in the epidermis and that the virus may disseminate to the skin via blood vessels [26].

Positive immunohistochemical staining in sweat glands confirms the findings by Recalcati et al. [27] and Liu et al., [28] who detected SARS-Cov-2 in sweat ducts and hypothesized sweat as a possible route of transmission of SARS-CoV-2.

Key strength of the present study is the high number of cases investigated as compared to the literature and the concurrent use of different methods including ddPCR, RNAscope and immunohistochemistry to demonstrate the presence of SARS-CoV-2 in lesional skin. Indeed, only one previous study chose more than one method to detect the virus in lesions of patients with COVID-19-associated cutaneous manifestations [19]. Another point of strength is the use of both positive (placenta and lungs) and negative (basal cell carcinoma and psoriasis) controls for viral detection by immunohistochemistry.
In conclusion, SARS-CoV-2 was detected only in 38% (20/52) of our skin samples, mainly by means of immunohistochemical staining for nucleocapsid antigen, without any association between the presence of SARS-CoV-2 in the skin and a specific cutaneous phenotype, suggesting that in most cases the pathogenesis of the skin lesions is associated to the activation of the skin immune system more than to a direct action against the virus. Lack of an efficient immune response against the virus, or higher viral loads *per se*, might represent critical factors leading to SARS-CoV-2 spread to the skin.

Figure and tables legends

Figure 1. (A) Immunohistochemical analysis and (B) in situ hybridization on placental syncytiotrophoblasts from a COVID-19 patient (positive control) showing positive staining for SARS-CoV-2 spike protein (purple signals) and spike mRNA (brown dots), respectively (40x magnification).
(C) Immunohistochemical analysis and (D) in situ hybridization of a representative chilblain-like acral lesion from patient IDXX showing positive staining for SARS-CoV-2 spike protein (purple signals) and spike mRNA (brown dots), respectively (40x magnification)
(E) Immunohistochemical analysis showing a positive staining for SARS-CoV-2 anti-nucleocapsid protein (40x magnification) in dermal sweat and sebaceous glands (inset in F, 100x magnification) and in small dermal vessels (inset in G, 100x magnification).
Table 1. Demographic and clinical features of the patients' cohort.
Table 2. Clinical data of patients who tested positive in immunohistochemistry, RNA-ISH and ddPCR

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