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Histopathological findings in systemic sclerosis-related myopathy: fibrosis and microangiopathy with lack of cellular inflammation

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Abstract

Objectives: The objective of this study was to identify specific histopathological features of skeletal muscle involvement in systemic sclerosis (SSc) patients.

Methods: A total of 35 out of 112 SSc-patients (32%, including 81% female and 68% diffuse scleroderma) presenting clinical, biological and electromyographic (EMG) features of muscle weakness, were included. Patients underwent vastus lateralis biopsy, assessed for individual pathologic features including fibrosis [type I collagen (Coll-I), transforming growth factor β (TGF-β)], microangiopathy [cluster of differentiation 31 (CD31), pro-angiogenic vascular endothelial growth factor A (VEGF-A), anti-angiogenic VEGF-A165b], immune/inflammatory response [CD4, CD8, CD20, human leucocyte antigens ABC (HLA-ABC)], and membranolytic attack complex (MAC). SSc biopsies were compared with biopsies of (n = 35) idiopathic inflammatory myopathies (IIMs) and to (n = 35) noninflammatory myopathies (NIMs). Ultrastructural abnormalities of SSc myopathy were also analyzed by transmission electron microscopy (TEM).

Results: Fibrosis in SSc myopathy (81%) is higher compared with IIM (32%, $p < 0.05$) and with NIM (18%, $p < 0.05$). Vascular involvement is dominant in SSc muscle (92%), and in IIM (78%) compared with NIM (21%, $p < 0.05$). In particular, CD31 shows loss of endomysial vessels in SSc myopathy compared with IIM ($p < 0.05$) and with NIM ($p < 0.01$). VEGF-A is downregulated in SSc myopathy compared with IIM ($p < 0.05$) and NIM ($p < 0.05$). Conversely, VEGF-A165b is upregulated in SSc myopathy. The SSc immune/inflammatory response suggested humoral process with majority (85%) HLA-ABC fibral neoexpression and complement deposits on endomysial capillaries MAC, compared with IIM ($p < 0.05$), characterized by CD4+/CD8+/B-cell infiltrate, and NIM ($p < 0.05$). TEM analysis showed SSc vascular alterations consisting of thickening and lamination of basement membrane and endothelial cell 'swelling' coupled to endomysial/perimysial fibrosis.

Conclusions: Fibrosis, microangiopathy and humoral immunity are predominant in SSc myopathy, even if it is difficult to identify specific histopathological hallmarks of muscle involvement in SSc, since they could be present also in other (IIM/NIM) myopathies.

Keywords: fibrosis, histopathology, microangiopathy, myopathy, systemic sclerosis

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Introduction

Systemic sclerosis (scleroderma, SSc) is an autoimmune connective tissue disease characterized by skin and internal organ fibrosis, coupled with widespread vascular pathology [Korn, 2001]. Skeletal muscle involvement in SSc was first considered in 1876, as a minor component of the disease associated with disuse [Medsger *et al.* 1968].

Nowadays, SSc skeletal muscle involvement turns out to be a common feature, with prevalence from 14% to 79% [Paik *et al.* 2014]. This variable prevalence results from the heterogeneous criteria used to define muscle involvement in SSc, including clinical, biological, electromyographic (EMG) and histological features of muscle weakness [Olsen *et al.* 1996]. In fact, SSc myopathy can occur for different reasons, such as result of non-autoimmune etiologies: malnutrition, disuse, or other neuromuscular disorders [Clements *et al.* 1978]. Another important issue related to defining specific criteria for recognizing 'SSc myopathy' as such an entity, is the exclusion (or not) of SSc overlap syndromes, since inflammatory myopathies such as polymyositis (PM) or dermatomyositis (DM) are a common feature in these syndromes [Pope, 2002]. Despite the absence of definite criteria for the diagnosis of 'SSc myopathy', researchers agree on the fact that SSc-muscle involvement is a negative prognostic feature impacting survival, and has also been associated with cardiopulmonary complications and even sudden cardiac death [Follansbee *et al.* 1993]. For these reasons, in the present study we tried to identify histopathological hallmarks of 'SSc myopathy' that could be unique for the disease itself and not common to other idiopathic inflammatory myopathies (IIM) or to other noninflammatory myopathies (NIM).

Methods

Patients and diagnostic procedures

We admitted 112 SSc patients in our institution from 2010 to 2015. Patients who had been diagnosed according to the 2013 American College of Rheumatology and European League Against Rheumatism (ACR/EULAR) diagnostic criteria for SSc [Van Den Hoogen, 2013], were enrolled. In total, 35 out of the 112 (32%, including 81% female and 68% diffuse scleroderma) who presented clinical, biological and EMG features of muscle involvement, were enrolled. Table 1 shows the main clinical and biological features of enrolled patients. Patients underwent biopsy of the vastus lateralis muscle. Specimens were frozen in liquid nitrogen-cooled isopentane and stored at −80°C until use. Cryostat sections were submitted to diagnostic routine histological and histochemical stains. SSc muscle biopsies were compared with (n = 35, site-matched) biopsies of patients with IIM, diagnosed following current clinicopathological criteria [Dalakas, 2010] [(n = 15 PM; n = 10 DM; n = 10 inclusion body myositis (IBM)], and to (n = 35, site-matched) biopsies of patients with NIM (n = 21 (A) metabolic myopathies, n = 14 (B) congenital myopathies): (A) n = 5 aspecific myopathic changes at biopsy; n = 4 statin associated rhabdomyolysis; n = 4 mitochondrial myopathy; n = 3 muscle glycogenosis; n = 5 steroid myopathy. (B) n = 2 calpainopathy; n = 3 mild dystrophinopathy; n = 2 myotonic dystrophy type 1; n = 2 myotonic dystrophy type 2; n = 2 facioscapulohumeral dystrophy; n = 3 oligosymptomatic familial hyperckemia). All subjects signed an informed consent, with allowance for scientific utilization of muscle samples for research purposes, in accordance to the principles of the 1975 Declaration of Helsinki (revised Hong Kong 1989) and the entire study protocol was approved by the institutional review board of the Ethics Committee of the University of Siena.

Histology and histoenzymatic stains

Cryostat 10 μm thick sections were submitted to routine hematoxylin–eosin and modified Gomori trichrome for morphological evaluation. Histoenzymatic stains for nicotinamide adenine dinucleotide (NADH) tetrazolium reductase, succinic dehydrogenase, cytochrome c oxidase, Periodic Acid Schiff (PAS) were also carried out.

Immunohistochemistry

Immunohistology for diagnostic routine analysis [CD4, CD8, CD20, human leucocyte antigens ABC (HLA-ABC), membranolytic attack complex (MAC)] (Dako, Glostrup, Denmark) was carried out on 7 μm thick cryostat sections on silane-coated slides (StarFrost; Knittel Gläser, Braunschweig, Germany). Vascular involvement was assessed by endothelial marker [cluster of differentiation 31 (CD31)] (Dako), pro-angiogenic vascular endothelial growth factor A (VEGF-A) (abcam, Cambridge, UK) and anti-angiogenic VEGF-A_{165b} (abcam). Muscle fibrosis was assessed by the analysis of type I collagen (Coll-I) (abcam) and transforming growth factor β (TGF-β) (abcam). All the reactions were performed by immunoperoxidase technique, by horseradish peroxidase (HRP)-labeled polymer (Dako), and 3,3'-diaminobenzidine (Sigma-Aldrich, Milan, Italy) for visualization. Negative controls were performed by the omission of the primary antibody.

Table 1. Clinical and biological features of enrolled patients.

SSc patients	lSSc	dSSc
Number of subjects (35)	11 (32%)	24 (68%)
Female sex, N (%)	7 (63%)	21 (87%)
Disease duration years (1st non-Raynaud's)	6.4 ± 4.8	7.9 ± 5.6
At the time of muscle biopsy (years)	4.5 ± 3.7	4.9 ± 3.7
ANA titer ⩾ 1:160	11 (100%)	24 (100%)
Anti-CENP-B	11 (100%)	0 (0%)
Anti-Scl-70	0 (0%)	24 (100%)
Anti-PM-Scl/Anti-Jo-1	0 (0%)	0 (0%)
Increased CK (>200 mcg/l)	5 (45%)	15 (62%)
Increased LDH (>190 u/l)	4 (36%)	15 (62%)
Increased aldolase (>7.5 u/l)	3 (27%)	9 (37%)
Increased myoglobin (>85 ng/ml)	3 (27%)	7 (29%)
Weakness (BMC testing <5)	11 (100%)	24 (100%)
EMG myopathic motor units	11 (100%)	24 (100%)

lSSc, limited systemic sclerosis; dSSc, diffuse systemic sclerosis; ANA, Anti-nuclear antibodies; CENP-B, Centromere Protein B; Scl-70, DNA Topoisomerase I; PM-Scl, exosome; Jo-1, histidyl tRNA synthetase; CK, Creatinine kinase; LDH, Lactate dehydrogenase; BMC, British Medical Council; EMG, Electromyography.
Pharmacological treatment: SSc patients enrolled in the study were in treatment with Iloprost (11 lSSc and 24 dSSc) for secondary Raynaud's phenomenon, with dual endothelin-1 receptor antagonist Macitentan (4 lSSc and 16 dSSc) for pulmonary arterial hypertension (PAH), with dual endothelin-1 receptor antagonist Bosentan (2 lSSc and 4 dSSc) for digital ulcers, with prokinetic and proton pump inhibitor agents (1 lSSc and 2 dSSc). It is important to underline that all the SSc patients enrolled in the study stopped treatments with low dose corticosteroids (<4–8 mg/die) and with immunosuppressive drugs at least three months before muscle biopsy.

Transmission electron microscopy analysis

Ultrastructural analysis of SSc muscle specimens was performed routinely after fixation in 2.5% glutaraldehyde for 3 h at 4°C, post fixation in 1% osmium tetroxide, and embedding in araldite. Ultrathin sections were stained with uranyl acetate and lead citrate and were observed at Philips EM-10 electron microscope (Huntsville, Alabama, USA).

Quantitative analysis on immunohistochemical slides

Morphometry was carried out by a Zeiss AxioPlan2 microscope equipped with AxioVision 4.6 software (Carl Zeiss Vision GmbH, Hallbergmoos, Germany). Microangiopathy ($CD31^+$ endomysial vessels, VEGF-A and VEGF-A_{165b}), inflammation (deposits of CD4, CD8, CD20 reactive cells), major histocompatibility complex (MHC)-I complex fibral neoexpression HLA-ABC, complement deposition MAC and fibrosis (Coll-I, TGF-β) were evaluated as histopathological parameters of muscle involvement. The density of $CD31^+$ endomysial vessels was expressed as a capillary to fiber ratio of muscle area, by counting vessels on the whole sections immunostained for CD31. VEGF analysis consisted of VEGF-A_{165b}/VEGF-A ratio of consecutive sections. Inflammatory (CD20, CD4, CD8), fibrotic (Coll-I, TGF-β) and complement deposition MAC scores were assessed on three randomly selected fields at 100× magnification by automatized colorimetric pixel evaluation, detecting the peroxidase reaction product. Each score was expressed as the $marker^+$ percentage of the total area.

Statistical analysis

Data were evaluated by GraphPad Prism 6® software for Windows. Analysis of variance (ANOVA) was performed by Kruskal–Wallis test for multiple groups. Significance was set at $p < 0.05$. Data are expressed as means ± standard deviations (SD).

Results

Histopathology

General myopathic changes, such as increased variability of fiber diameter, scattered atrophic fibers and occurrence of internalized nuclei were

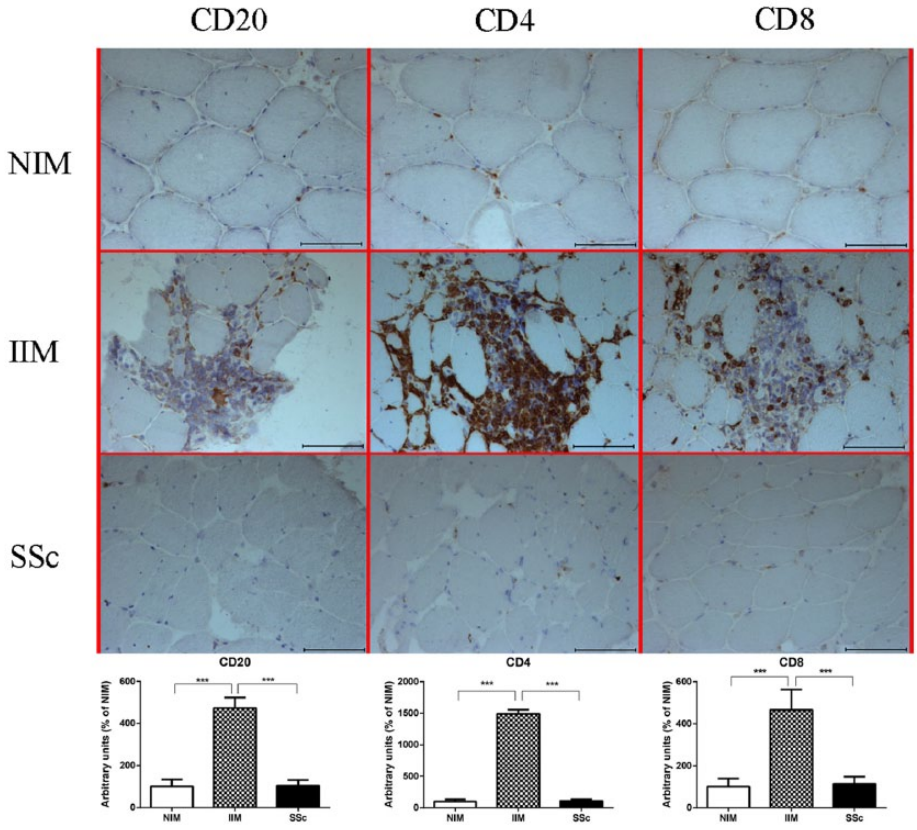

Figure 1. Representative images of immunohistological distribution (top) of CD20, CD4, CD8 on consecutive sections. Bars are set at 100 μm. Quantitative analysis (bottom) reveals that inflammatory cells are predominant in IIM group compared with NIM (***$p < 0.001$, Kruskal–Wallis) and SSc (***$p < 0.001$, Kruskal–Wallis) groups.
IIM, idiopathic inflammatory myopathies; NIM, noninflammatory myopathies; SSc, systemic sclerosis.

detected. Multifocal endomysial fibrosis and scattered dilated endomysial capillaries were also observed. Necrosis/regeneration was not prominent, with occasional single fiber necrosis in a minority of subjects.

Immunohistology and quantitative analysis

Immunohistology for diagnostic routine analysis (CD4, CD8, CD20, Figure 1; HLA-ABC, MAC, Figure 2) were reported with the relative quantitative analysis. The inflammatory cells result was present only in IIM group. Limited or no reactivity was recorded in NIM ($p < 0.001$) and SSc ($p < 0.001$) groups, while the sarcolemmal/cytoplasmic stain of HLA-ABC and MAC deposition on endomysial capillaries is statistically higher in SSc myopathy ($p < 0.01$; $p < 0.05$) and IIM ($p < 0.01$; $p < 0.01$) groups compared with the NIM group. Moreover, HLA-ABC and MAC stainings in the SSc myopathy group seem to be comparable to those of IIM group. In Figure 3, vascular involvement, represented by VEGF-A, VEGF-A_{165b}, CD31 and fibrotic component (represented by Coll-I and TGF-β) are reported. The VEGF-A_{165b}:VEGF-A ratio is statistically higher in SSc myopathy ($p < 0.01$) and IIM ($p < 0.05$) groups compared with the NIM group. The density of $CD31^+$ endomysial vessels is statistically decreased in the SSc myopathy group compared with the IIM ($p < 0.05$) and NIM ($p < 0.01$) groups. In addition, Coll-I and TGF-β reveal strong expression in activated endomysial and perimysial myofibroblasts of the SSc myopathy group, and also in the fibroblasts and in some inflammatory cells surrounding the necrotic fibers in the IIM group. Faint Coll-I and TGF-β immunoreactivity is recorded in the resident endomysial and perimysial fibroblasts of the NIM group. Quantitative analysis shows that Coll-I and TGF-β are statistically higher in the SSc-myopathy group compared with the IIM ($p < 0.05$; $p < 0.01$) and NIM ($p < 0.01$; $p < 0.01$) groups.

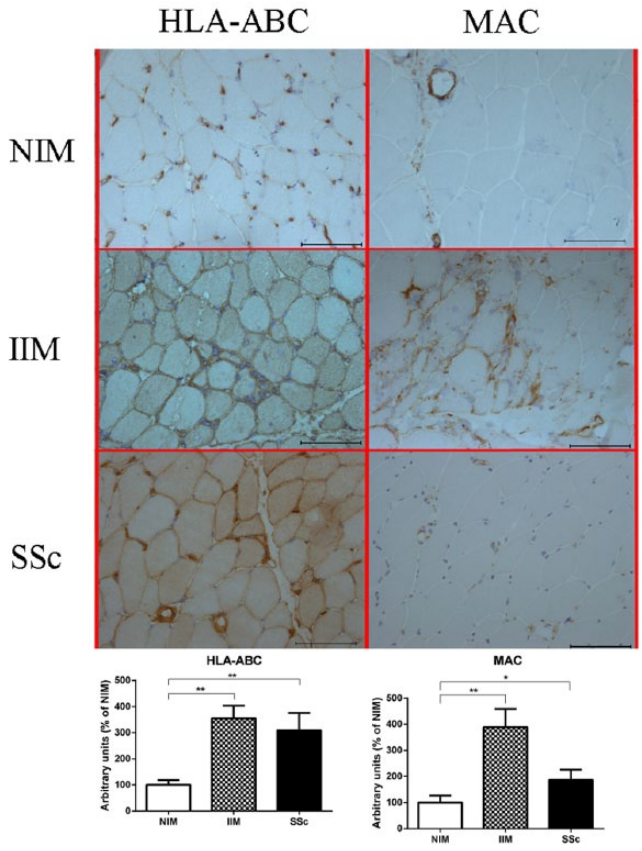

Figure 2. Immunohistological distribution (top) of HLA-ABC and MAC. Sarcolemmal and cytoplasmic HLA-ABC neoexpression is predominant in IIM and SSc groups. Necrotic fibers, identified by deposits of the terminal complex of complement, or MAC are particularly common to IIM group, while MAC deposits on capillary walls are particularly abundant in SSc group. Bars are set at 100 μm. Quantitative analysis (bottom): HLA-ABC is statistically higher in SSc myopathy (**$p < 0.01$, Kruskal–Wallis) and IIM (**$p < 0.01$, Kruskal–Wallis) groups compared with NIM group. MAC is highly upregulated in IIM (**$p < 0.01$, Kruskal–Wallis) and SSc-myopathy (*$p < 0.05$, Kruskal–Wallis) groups compared with NIM group.
HLA-ABC, human leucocyte antigens ABC; IIM, idiopathic inflammatory myopathies; NIM, noninflammatory myopathies; SSc, systemic sclerosis.

TEM analysis

Figure 4 shows a transmission-electron-microscopy (TEM) analysis of SSc muscle biopsies. Thickening and lamination of the basement membrane (middle), coupled to endothelial cell 'swelling' (left) are the main hallmark of SSc microangiopathy. Moreover, considerable endomysial fibrosis (right) with a large deposition of collagen is also observed.

Discussion

The most important problem when analyzing the literature of SSc-related muscle involvement is the total absence of specific criteria for the diagnosis of such an entity [Ranque *et al.* 2007]. This is because clinical, biological, EMG and histopathological features are very heterogeneous, and also because SSc-associated myopathy often includes several distinct entities with different pathogenetic mechanisms involved, and with distinct outcomes [Ranque *et al.* 2007; Morrisroe *et al.* 2015]. Even when focusing on muscle histopathological findings only, no clear-cut classification has emerged [Paik *et al.* 2015]. Therefore, novel insights on the immunopathological nature of SSc muscle involvement are needed. For this reason, in this study we tried to identify histopathological hallmarks that could be specific for SSc myopathy and not common to other IIM and NIM. Whether the association of IIM and SSc constitutes an overlap syndrome or should be considered a SSc manifestation still remains controversial [Troyanov *et al.* 2005; Bhansing *et al.* 2014]. We demonstrated that fibrosis (81%) and microangiopathy (92%) are the main

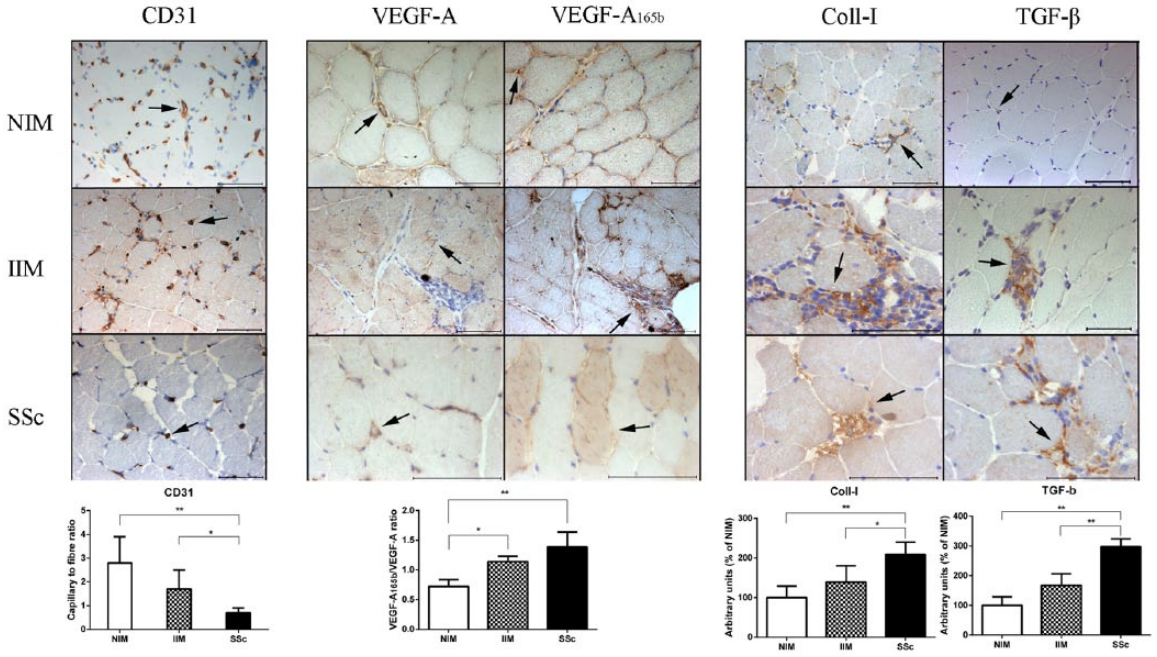

Figure 3. Immunohistology (top) and relative quantitative analysis (bottom) of CD31 (left), VEGF-A, VEGF-A_{165b} (middle) and Coll-I, TGF-β-A (right) in NIM, IIM and SSc groups, respectively. $CD31^+$ vessels (arrows) are abundant and surrounding myofibers in NIM group, while they decrease in particular in the SSc group. The density of $CD31^+$ endomysial vessels, expressed as capillary to fiber ratio of muscle area, is statistically decreased in SSc myopathy group compared with IIM (*$p < 0.05$, Kruskal–Wallis) and to NIM (**$p < 0.01$, Kruskal–Wallis) groups. VEGF-A and VEGF-A165b seem equally expressed in small endomysial vessels of NIM group; in SSc group, VEGF-A shows only the stain of endomysial vessels (arrows), while VEGF-A165b also shows a diffuse cytoplasmic stain of myofibers (arrows). The VEGF-A_{165b}:VEGF-A ratio is statistically higher in SSc myopathy (**$p < 0.01$, Kruskal–Wallis) and IIM (*$p < 0.05$, Kruskal–Wallis) groups compared with NIM group. Coll-I and TGF-β are expressed only by some resident endomysial fibroblasts (arrows) in NIM group, while they are expressed by both infiltrate cells and activated fibroblasts surrounding necrotic fibers in IIM group (arrows). In SSc group, endomysial activated fibroblasts or myofibroblasts are strongly reactive to Coll-I and TGF-β (arrows). Quantitative analysis demonstrated that endomysial expression of Coll-1 is significantly higher in SSc-myopathy group compared with IIM (*$p < 0.05$, Kruskal–Wallis) and to NIM (**$p < 0.01$, Kruskal–Wallis) groups. TGF-β is strongly upregulated in SSc myopathy group compared with IIM (**$p < 0.01$, Kruskal–Wallis) and NIM (**$p < 0.01$, Kruskal–Wallis) groups. Bars are set at 100 μm.
IIM, idiopathic inflammatory myopathies; NIM, noninflammatory myopathies; SSc, systemic sclerosis; TGF-β-A, transforming growth factor β-A; VEGF-A, pro-angiogenic vascular endothelial growth factor A; VEGF-A_{165b}, anti-angiogenic vascular endothelial growth factor A.

histopathologic hallmarks of SSc-related myopathy. We evidenced a reduced vascularization of muscle fibers through the analysis of CD31, and the prevalence of anti-angiogenic isoform VEGF-A_{165b}, as confirmed by other researchers, finding increased VEGF-A_{165b} plasmatic levels in SSc patients [Manetti *et al.* 2013]. However, these vascular alterations are not specific to SSc; they could be present also in other IIM myopathies, such as PM and DM, as has been well described in the literature [Volpi *et al.* 2013]. Regarding the inflammatory infiltrate, our study detected an absence of inflammatory cells in the majority of SSc cases (85%). MAC capillary deposits, compatible with a humoral immune process, suggest that endothelial injury and intimal proliferation may be mediated by complement-fixing antibodies [Evans *et al.* 1987]. Loss in muscle small endomysial vessels in SSc myopathy indicates a vasculopathy, with proliferative changes of the vessels wall, as evidenced by ultrastructural examination, as an early step in tissue damage, in analogy with skin and internal organs [Asano and Sato, 2015]. Nevertheless, HLA-ABC upregulation is considered an immunohistologic hallmark of inflammatory myopathies [Dalakas, 2010]. In view of our consistent finding of MHC-I fibral staining on SSc myopathy, with no relevant infiltrates or myonecrosis, a role of pleiotropic TGF-β might be speculated about as a negative

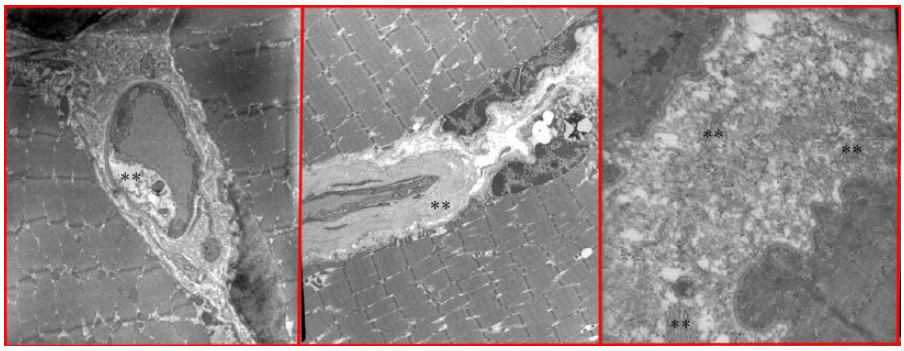

Figure 4. Transmission electron microscopy analysis of SSc muscle biopsies. (Left) Endothelial cell 'swelling' (asterisks) is one of the main hallmarks of microangiopathy (original magnification 6600×). (Middle) Thickening and lamination of the basement membrane (asterisks) are also recorded (original magnification 5200×). (Right) Extent endomysial fibrosis with large deposition of collagen (asterisks) (original magnification 15,500×).
SSc, systemic sclerosis

modulator of cell immunity [Morikawa *et al.* 2016], in reducing the extent of muscle inflammation, at least in the early phases of muscle involvement, and acting as a pro-fibrotic factor. This hypothesis is based on the documented upregulation of TGF-β in scleroderma lesional skin, where it induces the activation of myofibroblasts [Nikitorowicz-Buniak *et al.* 2015]. However, it must be remembered that the literature reported some cases of SSc myopathy with inflammatory cells, predominantly $CD8^+$ T lymphocytes with lower levels of $CD4^+$ cells, few B cells and no complement capillary deposits [Bhansing *et al.* 2014], as well as some cases with a majority of $CD4^+$ T cells and B cells and complement deposits [Ranque *et al.* 2007]. Therefore, data related to the inflammatory infiltrate and the nature of immune response seem controversial and different possible patterns are envisaged. In conclusion, our suggestion is to study and characterize each case of muscle involvement in SSc from the histological/immunohistological point of view, because of the heterogeneity of its manifestations. This advice is not simply speculative, but responds to the need to set up the suitable therapy for each case. In fact, high-dose steroids represent the first line treatment for all myositides except IBM [Dalakas, 2010]; in SSc myopathic patients, highly susceptible to renal crisis, corticosteroids at the lowest effective dose should be restricted to subjects with histologically proven inflammation. Further studies should follow to determine whether histopathologic findings in SSc-related myopathy could influence clinical outcomes of specific targeted therapies.

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Conflict of interest statement

The authors declare that there is no conflict of interest.

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