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Acro-osteolysis is associated with enhanced osteoclastogenesis and higher blood VEGF levels in systemic sclerosis

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Abstract

Objective—Bone resorption of distal phalanges or acro-osteolysis (AO) can develop in systemic sclerosis (SSc) causing pain and functional limitation. We investigated whether AO may be associated with abnormal osteoclastogenesis in SSc patients and whether hypoxia may be involved in this process.

Methods—Peripheral blood mononuclear cells (PBMCs) obtained from 26 SSc patients (11 with and 15 without AO) and 14 healthy controls (HC) were cultured in the presence of receptor activator of NF- κ B-ligand (RANKL) and macrophage colony-stimulating factor for 9 days. Tartrate resistant acid phosphatase (TRAP)+ multinucleated giant cells (MNGs) containing 3 or more nuclei were counted as osteoclasts (OCs). Plasma levels and effects of vascular endothelial growth factor (VEGF) on OC formation were evaluated.

Results—SSc patients with AO formed significantly more OCs after 9 days than did subjects without AO (142.4 ± 67.0 vs. 27.2 ± 17.6 MNGs/well, $p < 0.001$) or HC (18.7 ± 27.0 MNGs/well, $p < 0.001$). No significant difference in OC formation was noted between the patients without AO and HC. Plasma levels of VEGF were higher in SSc patients with AO compared to those without (142.4 ± 69.6 pg/mL vs. 88.1 ± 38.2 pg/mL, $p < 0.001$) or HC (54.2 ± 24.6 pg/mL, $p = 0.018$). Priming with VEGF-A for 24 hours significantly increased OC generation by 5.3 ± 1.9 fold ($p = 0.03$). The radiographic extent of AO was associated with increased OC formation (Spearman $\rho = 0.741$, $p = 0.01$).

Conclusion—Increased OC formation and higher VEGF levels may contribute to AO in SSc patients. Further studies are needed to elucidate whether targeting osteoclastogenesis may provide a specific therapeutic option for SSc-AO.

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Systemic sclerosis (SSc) is systemic autoimmune disorder characterized by excessive fibrosis of the skin and internal organs as well as by progressive vascular disease leading to chronic hypoxia in affected tissues.[1] Musculoskeletal involvement is common and can manifest with calcinosis, flexion contractures and in some cases with joint inflammation and erosions.[2] A subset of SSc patients can also develop a distinct pattern of bone resorption involving the distal portion of the fingers defined as acro-osteolysis (AO). [3-5] Less frequently this osteolytic process can be detected in the mandibular angle, vertebrae and epiphyseal segments of radius, ulna, and clavicles.[6] AO can extend proximally with extensive or even complete finger loss and digital telescoping, often resulting in severe functional limitation. The extra-articular location, the lack of inflammation and the absence of rheumatoid factor or anti-citrullinated peptide antibodies suggest that the mechanisms underlying SSc-related AO are different from those driving bone erosion in RA.

Osteoclasts (OCs) are multinucleated giant cells (MNGs) deriving from circulating monocytes or their bone marrow precursors in the presence of receptor activator of NF- κ B ligand (RANK-L) and macrophage colony-stimulating factor (M-CSF).[7] While they actively participate in physiologic as well as pathologic bone remodeling, their role in SSc-AO is not known to date.[8,9] The acral involvement and the consistent observation that SSc patients with AO tend to have worse vascular disease support the hypothesis that hypoxia could be a contributing factor in SSc-AO.[3-5] In keeping with this possibility, it has been noted that pathologic bone lysis tends to occur in the presence of low oxygen tension such as in sites of inflammation or tumor proliferation.[10] Experimental data have shown that hypoxia can drive formation of OCs from normal peripheral blood mononuclear cells and that vascular endothelial growth factor (VEGF), a potent angiogenic factor induced by hypoxic stimuli, can promote the generation and enhance survival of osteoclasts (OC). [11-14] The vasculopathy and associated chronic digital ischemia established in SSc may therefore contribute to the development of AO by promoting enhanced osteoclast formation and activation. In this study, we show that the presence of radiographically-confirmed AO in SSc patients is associated with increased propensity for osteoclast formation in the peripheral blood and higher plasma levels of VEGF.

MATERIALS AND METHODS

Patients

Patients evaluated at the Johns Hopkins Scleroderma Center were included in the study after providing written informed consent if they met the American College of Rheumatology preliminary criteria for the classification of SSc or had at least three of five features of CREST syndrome (calcinosis, Raynaud's phenomenon (RP), esophageal dysmotility, sclerodactyly, telangiectasias).[15] The study was approved by the Johns Hopkins institutional review board. Demographic and clinical data including age, sex, ethnicity, smoking status, disease duration defined from onset of Raynaud's phenomenon (RP) as well as first non-RP symptom, scleroderma subtype, autoantibody status and medication usage were recorded at the time of the visit. Subjects were subdivided as having diffuse or limited scleroderma based on the extent of their skin involvement and the modified Rodnan skin score (mRSS) was used to quantify its severity.[16,17] Ischemic digital loss was defined as

amputation of a portion or the entire finger or toe following an irreversible ischemic event at any time in their disease course. The presence of ischemic (non-traumatic) digital ulcers was assessed on the tips or on the side of the fingers together with that of pitting scars, defined as pinhole-sized digital concave depressions with hyperkeratosis. Raynaud's activity and the presence of digital ischemia was determined using a previously published severity score: 0-No Raynaud's, 1-Raynaud's with/without vasodilator required, 2-Digital Pitting Scars, 3-Digital Tip Ulcerations, 4-Digital Gangrene.[18] Lung involvement was determined based on abnormal pulmonary function tests (PFTs) and measurement of forced vital capacity (FVC) and single-breath carbon monoxide transfer factor (DLCO) were calculated according to the American Thoracic Society recommendations.[19] Values for spirometry were referenced to those of the National Health and Nutrition Examination Survey/Hankinson et al study and the values for DL_{CO} were reference to those reported by the Knudson et al. [20,21] The presence of restrictive lung disease (RLD) was defined as a FVC less than 80% of standardized predicted. Echocardiographic evidence of pulmonary hypertension (ECHO-PH) was defined as an estimated right ventricular systolic pressure determined by Doppler echocardiography greater than 45 mm Hg in the absence of clinical evidence of congestive heart failure or thromboembolic diseases. The presence of AO was determined by the examining clinician as shortening of one or more distal phalanx not related to amputation secondary to trauma or irreversible finger tip infection. All enrolled subjects with or without AO underwent confirmatory bilateral hand x-rays.

Radiographic image analysis

Radiography (anterior-posterior and oblique views) of both hands obtained in study patients were independently reviewed by two experienced musculoskeletal radiologists (JAC and FDG) and evaluated for the presence of AO and soft tissue calcinosis. The scoring system for radiographic evidence of AO in each digit was as follow: 0 = none; 1 = minimal/doubtful; 2 = evident less than 50% of the tuft; 3 = evident more than 50% of the tuft. A composite score for AO ranging from 0 to 30 (maximum score 3 for every finger of both hands) was calculated. A consensus was reached for the scores assessed in each digit of both hands. Soft tissue calcinosis was defined as absent or present in any of the fingers.

Cell and plasma preparation

Peripheral blood mononuclear cells (PBMCs) were isolated from heparinized peripheral venous blood by density gradient centrifugation using Ficoll-Paque (GE Healthcare, NJ, USA). Cell viability was assessed with trypan blue dye exclusion. Plasma samples were isolated from blood collected in sodium citrate-containing tubes within three hours from venipuncture. After centrifugation for 10 minutes at 1,500 x g plasma was collected, further spun at 15,000g × 5 minutes and immediately stored at -80°C.

Determination of circulating osteoclast precursor cells

PBMCs (2×10^5 per well) were cultured in OPTI-MEM® I (Gibco/Invitrogen, Grand Island, NY, USA), supplemented with 10% heat-inactivated fetal bovine serum, 1% penicillin and 1% streptomycin in the presence of 100 ng/ml RANK-L (R&D Systems, Minneapolis, MN, USA) and 25 ng/ml M-CSF (R&D Systems, Minneapolis, MN, USA) in a 96 well plate in 5% carbon dioxide at 37°C. On day 9, cells were fixed with 3% formaldehyde and stained

for expression of TRAP per the manufacturer's protocol (Sigma, St Louis, MO, USA). TRAP+ cells with three or more nuclei were counted as OCs under light microscopy Olympus CKX41 using magnification X 20. For priming with VEGF, PBMCs were pre-incubated with 10 ng/ml VEGF-A (PeproTech, NJ, USA) for 24 hours. After priming, the PBMCs were washed repeatedly with PBS and then cultured in the OC promoting media.

Pit formation assay

PBMCs were cultured in the same conditions described above using Corning® Osteo assay surface 96 well multiple plates (Corning Life Sciences, Tewksbury MA, USA), which are coated with inorganic crystalline calcium phosphate. After removing the medium, cells were lysed using 10% bleach solution for 5 minutes at room temperature. The inorganic coating and dissolved pits were contrasted by 1% toluidine blue. Pits were observed at 10× magnification.

VEGF measurement

Plasma levels of VEGF were quantitated using an electrochemiluminescence platform (Meso Scale Discovery, Gaithersburg, MD, USA) following manufacturer's instructions.

Statistical analysis

Demographic and clinical characteristics between patients with and without AO were compared using Mann-Whitney test for continuous and Fisher exact test for categorical variables. One way analysis of variance by ranks (Kruskal-Wallis test) was used for group comparisons and post-hoc analyses were adjusted for multiple comparisons with Dunn's correction. To assess the effect of VEGF on OC formation, Wilcoxon signed-rank tests were used. Linear associations were analyzed using Spearman rho. P value of 0.05 or less is considered statistically significant. All calculations were performed using GraphPad Prism version 6.0 for Mac OS X, GraphPad Software, San Diego, California, USA.

RESULTS

A total of 26 patients (11 SSc with and 15 without AO) were enrolled. These were mainly middle aged (53.6 ± 9.9 years), Caucasian (73%), women (96 %) with mean disease duration of 11.6 ± 13.4 years from RP onset (8.7 ± 10.1 years from 1st non-RP symptom). SSc patients with AO exhibited significantly higher RP severity (1.7 ± 0.8 vs. 1.0 ± 0.4 , $p=0.006$), higher prevalence of digital pits (45.5% vs 6.7%, $p=0.02$) and more frequent calcinosis (72.7% vs. 26.7%, $p=0.042$). The two groups were otherwise similar with regards to the other demographic and clinical characteristics, including smoking history, SSc skin type, disease duration, autoantibodies, heart and lung involvement and use of medications as summarized in Table 1.

Osteoclastogenesis is increased in SSc patients with AO

To determine whether the propensity to generate osteoclasts is increased in SSc patient with AO, we measured the frequency of osteoclast formation in the peripheral blood of study subjects. After 9 days in culture, PBMCs from healthy controls (HC) could generate only few, small MNGs (Fig. 1A, left panel). In contrast, SSc patients PBMCs formed numerous

large TRAP+ MNGs with numerous nuclei (Figure 1A, right panel). As shown in Figure 1B, the osteoclastic activity of these blood-derived MNGs was confirmed as they exhibited the distinct ability to digest bone matrix (Figure 1B). The mean pit size generated by MNGs derived from SSc PBMCs was on average 15 folds larger in than those from HC PBMCs (Supplementary Figure 1). PBMCs obtained from SSc patients with AO generated significantly more TRAP+ MNGs compared to SSc patients without AO (142.4 ± 67.0 vs. 27.2 ± 17.6 MNGs/well, $p < 0.001$) and HC (18.7 ± 27.0 MNGs/well, $p < 0.001$) (Figure 2). Number of generated OCs did not differ significantly between SSc subjects without AO and HC.

VEGF is increased in SSc-AO and enhances osteoclastogenesis

Several studies have previously reported a robust association between AO and severe underlying vascular disease.[3-5] To better elucidate whether stimuli deriving from chronic ischemia may be implicated in the development or progression of AO, we measured blood levels of VEGF, which is strongly induced by hypoxia and known to be upregulated in SSc. [22-24] As shown in Figure 3A, VEGF plasma levels were significantly higher in SSc patients with AO compared to those without (142.4 ± 69.6 pg/mL vs. 88.1 ± 38.2 pg/mL, $p=0.005$) or HC (54.2 ± 24.6 pg/mL, $p<0.001$). In addition, the number of MNGs generated from PBMCs exhibited a trend for association with VEGF plasma concentration (Spearman $\rho=0.386$, $p=0.053$) (Figure 3B). Interestingly, a subset of SSc subjects with AO showed a high propensity for osteoclast formation even in the presence of low levels of VEGF (Figure 3B). Experimental evidence has shown that VEGF can drive osteoclast differentiation and survival as well as directly enhance osteoclastic bone resorption.[12,13,25] In order to confirm the ability of VEGF to augment osteoclastogenesis, PBMCs from 5 consecutive HC were primed with VEGF-A at 10 ng/ml for 24 hours and then cultured in OC-promoting cytokine condition. The number of MNGs increased significantly after VEGF-A priming ($p=0.032$), with an average OC formation enhanced by 5.3 ± 1.9 fold ($p=0.0018$) (Figure 3C and D). A similar response was observed in 4 SSc patients without AO.

Severity of acro-osteolysis is associated with enhanced osteoclastogenesis

Radiography of both hands was obtained in each study subject to confirm the presence of AO (Fig. 4A). As the severity of distal tuft resorption can vary significantly even among digits in the same hand, a composite score for AO (range 0-30) was calculated as outlined in the Methods section. While SSc patients without clinical AO did not have any radiographic evidence of acral bone damage (AO score=0), the mean AO score in the active AO group was 18.5 ± 10.0 ($p<0.001$). Remarkably, the individual AO scores exhibited a significant association with the propensity to osteoclast formation (Spearman $\rho=0.741$, $p=0.011$) (Figure 4B) but not with VEGF levels. In addition, medical treatment with immunosuppressive agents or vasodilators was not associated with osteoclast formation or blood VEGF levels (Supplementary Table 1).

DISCUSSION

In the present study we provide strong evidence that the propensity for osteoclast formation is increased in the peripheral blood of SSc patients with acro-osteolysis (AO) and that this is

associated with higher plasma levels of VEGF as well as more advanced digital bone resorption.

AO represents a rare but unique musculoskeletal manifestation in a sizable subset (20-25%) of SSc patients and can be associated with significant disability, functional limitation and disfigurement. [2-5] The pathogenesis of AO is not well understood, but several studies have shown a strong clinical association between SSc vascular disease and AO. [3-5] Our study identifies a possible pathogenetic link between SSc vasculopathy and the development of AO. The positive association between osteoclastogenesis and VEGF levels in SSc patients indicates that chronic or recurrent hypoxia may enhance OC formation and their osteolytic activity within poorly perfused anatomical districts such as the digital tufts, eventually resulting in osteolysis. Beyond promoting angiogenesis, VEGF can augment osteoclastogenesis; it exerts chemotactic stimuli on monocyte precursors and induces their differentiation into osteoclasts in the presence of RANKL.[26] Further, it promotes direct activation and survival of mature osteoclasts, suggesting that hypoxia with VEGF production may be relevant in advancing osteolysis.[14] In this study, VEGF showed the ability to increase osteoclast formation by 5 to 6 folds both in healthy and SSc patients. However, our data were limited as the response to VEGF was tested only in SSc patients without AO. Interestingly, despite VEGF priming, the absolute number of MNGs generated from healthy controls PBMCs was lower than that of AO subjects, implicating that other factors besides VEGF may amplify osteoclastogenesis in SSc. This in part confirmed by the detection of a substantial number of SSc subjects with AO exhibiting higher propensity for osteoclast formation even in the presence of relatively lower levels of VEGF. Further investigation is needed to determine whether the enhanced osteoclastogenesis observed in SSc may be due only to an increased sensitivity to induction by RANK-L and M-CSF, or whether it may also results from a higher frequency of circulating precursors.

Osteolysis can be observed in the context of carpal tunnel syndrome (CTS), diabetic neuropathy and post-traumatic local autonomic dysfunction, conditions where impaired digital perfusion may result from failure of autonomic fibers to regulate the vasomotor balance of the digital arterioles. [27-29] CTS-like entrapment or more distal nerve compressions may develop in SSc hands secondary to deep fibrosis, flexion contractures and subcutaneous calcinosis.

Several reports have shown that SSc patients exhibit significantly lower bone density than HC and that their risk for osteoporosis and fractures is increased independently of corticosteroids use.[30-32] It would be of interest further investigating whether osteoporosis in SSc may be mechanistically linked to enhanced osteoclast formation driven by chronic or recurrent hypoxia. It is tempting to speculate that systemic hypoxia may contribute to osteoporosis, whereas a local hypoxic state within fingertips may sustain AO.[33] Abnormalities of calcium and phosphorus metabolism have been implicated in AO by Braun-Moscovici et al., who have found that a large proportion of patients with AO (but none of those without distal tuft resorption) exhibit significantly increased levels of parathyroid hormone (PTH) in presence of normal serum electrolytes and renal function.[34] Calcium absorption is decreased in SSc due to gastrointestinal disease, use of proton pump inhibitors and lower vitamin D levels and can result in secondary hyperparathyroidism

which can further increase bone loss. While our study did not address the effect of calcium, phosphate, PTH and vitamin D levels on osteoclast formation, this should be thoroughly evaluated in future studies.

The clinical observation that not all the patients with severe vascular disease develop AO suggests that other factors beyond hypoxia may play critical roles to initiate and/or sustain AO in SSc. While specific inflammatory events such as those leading to articular bone erosions in RA do not appear to be involved in SSc-AO, a perturbed immune function may contribute to AO by altering the priming and differentiation of myeloid precursor cells towards osteoclasts in the peripheral circulation.[35-38] In addition, direct or VEGF-mediated osteoclast activation may also be driven by increased production of cytokines over-expressed in SSc such as IL-1 β , IL-6, IL-17, and TGF- β . [23, 39, 40]

The limited knowledge about disease-specific pathogenetic pathways involved with SSc-AO has limited the ability to identify targeted treatments. The absence of obvious inflammation at the sites of AO questions the benefit of immunomodulatory therapies such as those effective for inflammatory joint disease (i.e. corticosteroids or anti-TNF agents). Intuitively, restoring proper digital blood flow should be a reasonable approach to manage AO. In reality, assertive vasodilatation does not seem to stop progression in severe cases. This may be due to the fact that SSc vasculopathy is chronic and microcirculation tends to be permanently damaged with poor reversibility, particularly in the distal portion of the digits. The presence of increased osteoclast activation in AO would support the use of anti-resorptive therapy. While bisphosphonates can prevent bone loss by inhibiting OC function directly and through downregulation of VEGF activation, our personal experience with these medications in SSc-AO patients has not been satisfactory.[41, 42] Similarly, the use of anti-resorptive agents in hereditary forms of AO has shown benefit with regards to treating osteoporosis but did not prevent progression of digital osteolysis.[43, 44] Instead, the use of RANKL inhibitors such as denosumab, which specifically target osteoclastogenesis, alone or in combination with vasodilators may prove to be a more successful strategy to control AO in SSc, particularly in consideration of the enhanced systemic tendency to form OCs detected in subjects of this investigation. [45-47]

Our study has several limitations. It was not possible to investigate osteoclastogenesis at the sites of acral bone resorption. Another important limitation is the lack of insight about the status of local digital circulation. Formal nailfold capillaroscopy was not conducted in our patients, but would have allowed a more precise characterization of vascular abnormalities in association with AO and a better definition of their relevance for the disease process. The clinical and radiographic course of SSc-AO is variable as the degree of osteolysis can stabilize in some patients while in others can progress towards complete loss of distal phalanxes and in some cases even to digital telescoping.[2] While our data show that peripheral blood OC formation and VEGF levels are associated with AO severity, prospective studies are needed to establish whether these abnormalities can also predict disease progression.

In conclusion, AO is associated with an increased propensity of peripheral blood cells to form osteoclasts and this seems to be potentiated by high VEGF levels. Improving hypoxia

and targeted inhibition of osteoclastogenesis may be effective in preventing AO from progressing.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1. Significant osteoclast formation can be induced in PBMCs obtained from SSc patients Peripheral blood mononuclear cells (PBMCs) were cultured in the presence of 25 ng/ml M-CSF and 100 ng/ml RANKL for 9 days. **A.** Comparison of TRAP+ multinucleated giant cells (MNGs) generated from PBMCs of healthy controls (left panel) and SSc patients with acro-osteolysis (right panel). **B.** Degradation of bone matrix was selectively induced by MNGs derived from SSc patients (left panel) compared to controls (right panel). Original magnification $\times 10$.

Figure 2. Increased osteoclast formation in SSc patients with acro-osteolysis

Distinct osteoclast formation from PBMCs obtained from health controls (n= 14) and SSc patients without (n=15) or with AO (n =11). TRAP+ MNGs were counted after 9 days of PBMCs incubation with RANKL and M-CSF.

AO, acroosteolysis; MNG, multinucleated giant cells; PBMCs, peripheral blood mononuclear cells; ns, not significant; SSc, systemic sclerosis; TRAP, tartrate resistant acid phosphatase

Figure 3. VEGF levels in SSc patients with AO and its association with OC formation

A. Plasma levels of VEGF in SSc patients with acro-osteolysis (AO) compared to SSc subjects without AO and healthy controls. **B.** Linear association between plasma VEGF levels and number of generated TRAP+ MNGs. **C-D.** Effects of VEGF-A priming on osteoclastogenesis: absolute TRAP+ MNGs formation from healthy PBMCs (C) and fold change from baseline (D). AO, acroosteolysis; MNG, multinucleated giant cells; SSc, systemic sclerosis; TRAP, tartrate resistant acid phosphatase; VEGF, vascular endothelial growth factor.

Figure 4. Association between degree of AO and osteoclastogenesis

A. Plain radiograph of a right hand revealing significant resorption of the distal phalanges in SSc patient with acro-osteolysis (AO). **B.** Linear relationship between the radiographic extent (severity) of AO and number of formed TRAP+ MNGs in each individual SSc patient.

*A composite AO score (range 0-30) was generated by grading radiographic AO severity in each digit as: 0 = none; 1 = minimal/doubtful; 2 = evident less than 50% of the tuft; 3 = evident more than 50% of the tuft.

Table 1

Demographic and clinical characteristics of SSc patients.

Variables	Total (n=26)	No AO (n=15)	AO (n=11)	P value
Age, years *	53.6 ± 9.9	56.1 ± 9.2	50.2 ± 10.2	0.194
Female	25 (96.1)	15 (100)	10 (90.9)	0.423
Race				
Caucasian	19 (73.1)	12 (80.0)	7 (63.6)	0.478
African American	6 (23.1)	3 (20.0)	3 (27.3)	
Other	1 (3.8)	0.0	1 (9.1)	
Smoking history				
Never	16 (61.5)	10 (66.7)	6 (54.5)	
Past	8 (30.8)	4 (6.7)	4 (36.4)	0.834
Current	2 (7.7)	1 (6.7)	1 (9.1)	
SSc types				
Limited	12 (46.2)	8 (53.3)	4 (36.4)	0.453
Diffuse	14 (53.8)	7 (46.7)	7 (63.6)	
Disease duration (from 1st non-RP symptom), years *	8.7 ± 10.1	9.3 ± 11.3	7.9 ± 8.7	1.000
Disease duration (from RP onset), years *	11.6 ± 13.4	14.2 ± 15.6	8.1 ± 9.2	0.346
mRSS (0-51) *	8.7 ± 11.0	10.5 ± 12.5	6.4 ± 8.6	0.459
RP severity score (0-4) *†	1.3 ± 0.7	1.0 ± 0.4	1.7 ± 0.8	0.006
Digital ulcerations	2 (7.7)	0 (0)	2 (18.2)	0.169
Digital pits	6 (23.8)	1 (6.7)	5 (45.5)	0.020
Digital loss	1 (3.8)	1 (6.7)	0 (0)	0.382
Calcinosis	12 (46.1)	4 (26.7)	8 (72.7)	0.042
Pulmonary function				
FVC (% predicted) *	78.7 ± 16.0	79.7 ± 13.1	77.4 ± 20.0	0.585
DLCO (% predicted) *	77.5 ± 17.3	76.3 ± 16.8	79 ± 18.8	0.754
RLD	11 (42.3)	6 (40.0)	5 (45.5)	1.000
eRVSP *	32.5 ± 21.2	29.0 ± 14.4	37.2 ± 28.1	0.376
ECHO-PH	3 (12)	1 (6.7)	2 (18.2)	0.556
Autoantibodies				
ANA	26 (100)	15 (100)	11 (100)	
Scl-70	11 (42.3)	6 (40.0)	5 (45.5)	1.000
ACA	10 (38.5)	6 (40.0)	4 (36.4)	1.000
RNA-polymerase 3	3 (11.5)	1 (6.7)	2 (18.2)	0.556
Medications				
Immunosuppressants ††	9 (34.6)	6 (40.0)	3 (27.3)	0.683
Vasodilators ¶	13 (50.0)	6 (40.0)	7 (63.6)	0.428
Statins	3 (11.5)	2 (13.3)	1 (9.1)	1.000

Variables	Total (n=26)	No AO (n=15)	AO (n=11)	P value
Aspirin	1 (3.8)	1 (6.7)	0	1.000

All values are given as number (%) unless otherwise specified.

ACA, anticentromere antibody; ANA, antinuclear antibody; AO, acro-osteolysis; DLCO, diffusion capacity of lung for carbon monoxide; eRVSP, estimated right ventricular systolic pressure; FVC, forced vital capacity; mRSS, modified Rodnan skin score; RLD, restrictive lung disease; RP, Raynaud's phenomenon; eRVSP, right ventricular systolic pressure estimated by echocardiography; Scl-70, topoisomerase I; SSc, systemic sclerosis. ECHO-PH, pulmonary hypertension.

* Mean $\pm$ SD.

[†] RP severity score is reported as previously defined by Medsger et al.[18]

^{††} Use of immunosuppressants includes one or more of cyclophosphamide, mycophenolate, methotrexate, or prednisone.

[¶] Use of vasodilators includes one or more of calcium channel blockers, phosphodiesterase-5 inhibitors and prostacyclin.