Association of endothelial to mesenchymal transition and cellular senescence with fibrosis in skin biopsies of systemic sclerosis patients: a cross-sectional study

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Abstract

Objective

Fibrosis is the dominant hallmark of systemic sclerosis (SSc). Several mechanisms have been proposed to drive the disease process, but how these relate to skin fibrosis is poorly understood.

Methods

We performed a cross-sectional study on archival skin biopsies from 18 SSc patients and four controls. Dermal fibrosis and inflammatory cell infiltration were scored in HE and Masson's Trichrome-stained sections. The presence of senescence was defined by P21 and/or P16 positivity in Ki-67 negative cells. Endothelial to mesenchymal transition (EndMT) was identified by co-localisation of CD31 and α-SMA in immunofluorescent double-stained sections, and by an enclosure of ERG positive endothelial cell nuclei by α-SMA stained cytoplasm in immunohistochemical double staining.

Results

The histological dermal fibrosis score of SSc skin biopsies was correlated with the modified Rodnan skin score (rho 0.55, p=0.042). Staining for markers of cellular senescence on fibroblasts was correlated with fibrosis score, inflammatory score, and CCN2 staining on fibroblasts. Moreover, EndMT was more abundant in skin from patients with SSc (p<0.01) but did not differ between groups with different fibrosis severity. The frequency of these EndMT features increased with the abundance of senescence markers and CCN2 on fibroblasts and dermal inflammation.

Conclusion

EndMT and fibroblast senescence were more abundant in skin biopsies from SSc patients. This finding indicates that both senescence and EndMT are involved in the pathway leading to skin fibrosis and might be valuable biomarkers and/or possible targets for novel therapeutic interventions.

Key words

connective tissue diseases, systemic sclerosis, fibrosis, scleroderma, cellular senescence

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Introduction

Systemic sclerosis (SSc) is an autoimmune disease characterised by a triad of inflammation, vasculopathy and fibrosis. Fibrosis, particularly prominent in skin and lungs, is a pivotal process in SSc (1). Fibrogenesis is mediated by the activation and proliferation of fibroblasts to myofibroblasts which leads to excessive extracellular matrix production. Several mechanisms may be involved in fibrogenesis, including response to inflammation and vasculopathy, but its pathogenesis still remains poorly understood.

As mentioned earlier, endothelial to mesenchymal transition (EndMT) and cellular senescence may be involved in the pathogenesis of SSc (2, 3). EndMT is a transition process of endothelial cells acquiring more mesenchymal characteristics, including expression of α-SMA and various extracellular matrix (ECM) molecules (2). In physiological and pathological conditions, EndMT can be induced by transforming growth factor beta (TGF-β), cellular communication network factor 2 (CCN2, also known as CTGF or connective tissue growth factor), and endothelin-1 (4, 5). Both CCN2 and TGF-β are also phenotypic markers in SSc and contribute to the cellular senescence-associated secretory phenotype (SASP) that stimulates fibrosis and inflammation (6-8). Cellular senescence is a permanent status of cell cycle arrest. It can occur in response to various stimuli, including telomere shortening, oxidative stress, genotoxic injury, and inflammation (6, 9). Dermal fibroblasts from patients with SSc have been shown to have less abundant mini-chromosome maintenance helicase proteins, lower autophagic capacity, and increased senescence features, including β-galactosidase activity and SASP (10, 11). To further explore the potential of histopathological assessment of skin biopsies for EndMT and cellular senescence features in SSc as biomarkers of fibrosis severity and identification of novel therapeutic targets, we studied archival skin tissue biopsies from SSc patients and controls in relation to skin fibrosis, reflected by the modified Rodnan skin score (mRSS).

Materials and methods

Patients

We performed a cross-sectional biobank study on formalin-fixed paraffin-embedded (FFPE) skin biopsies from patients meeting the ACR/EULAR 2013 classification criteria for SSc. Demography, comorbidity, medication, autoantibodies, disease duration, disease subset and mRSS at the time of biopsy were retrieved from medical records. Healthy controls were acquired from normal skin in adult resection material for unrelated indications. This study was approved by the institutional review board at University Medical Centre Utrecht (TcBio 22-014).

Histopathology

Slides were observed under a photo microscope (Nikon Eclipse E800) or fluorescence microscope (Leica DM 5500 B). Fibrosis and inflammatory cell infiltration of the dermis were scored in haematoxylin and eosin stain (HE) and Masson's Trichrome stained 3 μm sections from FFPE skin biopsies. Fibrosis severity was semi-quantified by the extent of dermal fibrosis and scored from one to three (12), and fibrosis activity was estimated from CCN2-staining (see below). Dermal inflammatory score was estimated from lymphocyte infiltration in the dermis and scored from zero to two. All semi-quantitative scores were evaluated independently in the superficial and deep dermis. The resulting score was recorded by agreement of two researchers (MRD and YHC). Immunohistochemical staining for P16, P21, and CCN2 in fibroblasts and endothelial cells was scored as absent (0), weak (1), or strong (2). The percentage of P16/P21 positive fibroblasts was counted in 10 random high-power-fields. The presence of senescence was defined as P21 or P16 positive without co-localised Ki-67 (13). EndMT was detected by co-localisation of CD31 and α-SMA by immunofluorescence double staining (α-SMA-CD31 positive) and enclosure of ERG positive endothelial cell nuclei by α-SMA stained cytoplasm in immunohistochemically double-stained (α-SMA-ERG positive) sections. EndMT was quantified by the percentage of vessel cross-sections de-

tected EndMT feature in dermis. The endothelial cells of both blood vessel and lymphatic ducts express CD31 and ERG, whereas only the lymph endothelial cells express D2-40 (podoplanin) (14). Therefore, numbers of lymph vessels/mm^2 were determined in D2-40 stained sections.

Primary antibodies used were rabbit anti-P21 (Cell Signaling Technologies 2947, 1:100 dilution), mouse anti-P16 (Immunologic VWRKILM0632-C05, 1:800 dilution), rabbit anti-Ki-67 (Thermo Scientific RM9106S, 1:400 dilution), rabbit anti-CCN2 (Cell Signaling Technology 86641, 1:600 dilution), mouse anti-α-SMA (Sigma A2547, 1:8000 dilution), rabbit anti-α-SMA (Abcam ab5694, 1:1000 dilution), rabbit anti-CD31 (LSBio LS-B4737, 1:50 dilution), rabbit anti-ERG (Dako M7314, 1:200 dilution), and mouse anti-D2-40 (Biolegend 916602, 1:400 dilution).

Digital analysis

The slides were scanned and imported in QuPath 0.4.1 (15). The figures were colour deconvoluted with vectors in haematoxylin, target staining (immunohistochemistry staining and blue in Masson's Trichrome staining) and residual (unwanted or confounding colour, including Ki-67 in the double staining, debris and haemosiderin deposition). After manually annotating the dermis, excluding hair follicles, muscle, glands, fat and vessels (with a wall of more than 3 cell layers), the percentage of positive staining pixels in the annotation was determined by a pixel classifier.

Statistical analysis

The difference of continuous variables between groups was determined using Kruskal-Wallis test and Wilcoxon rank-sum test. The correlation between ordinal and/or continuous variables was examined with Spearman's rank correlation rho. A p-value <0.05 was considered statistically significant.

All statistical analyses were performed using R 4.0.3.

Results

Patient characteristics

In total, skin biopsies from 18 SSc patients were identified in the archive of the Department of Pathology of the UMC Utrecht and investigated together with skin samples from four adult healthy controls. The healthy controls had a median age of 38 (34–47) years, and three of the four (75%) healthy controls were females. The control skin was taken two from lower limb and two from trunk. Patients' characteristics are summarised in Table I.

Table I. Clinical features of 18 patients with systemic sclerosis.

	All, n=18	Limited SSc, n=13	Diffuse SSc, n=5
Age, median (IQR)	47 (36—56)	46 (36—54)	49 (36—68)
Female, n (%)	11 (61)	7 (53)	4 (80)
Disease duration month, median (IQR)	5.5 (0.0—29.0)	5 (0—26)	6 (2—30)
Biopsy site, n (%)			
Upper limb	3 (17)	2 (16)	1 (20)
Trunk	6 (33)	5 (38)	1 (20)
Lower limb	8 (44)	5 (38)	3 (60)
mRSS, median (IQR)	7 (3—12)	3 (2—10)	27 (22—30)
Autoantibodies, n (%)			
Anti-topoisomerase I antibody	3 (17)	1 (8)	2 (40)
Anti-RNA polymerase III antibody	2 (11)	1 (8)	1 (20)
Anti-centromere antibody	3 (17)	3 (23)	0
Negative on autoantibodies	1 (6)	1 (8)	0
Immunosuppressants, n (%)			
Mycophenolate mofetil	1 (6)	0	1 (20)
Methotrexate	2 (11)	1 (8)	1 (20)
Disease complications, n (%)			
ILD	2 (11)	1 (8)	1 (20)
PAH	3 (17)	2 (15)	1 (20)
Digital ulcers	3 (17)	2 (15)	1 (20)

SSc: systemic sclerosis; IQR: interquartile range; mRSS: modified Rodnan skin score; ILD: interstitial lung disease; PAH: pulmonary arterial hypertension.
Biopsy site was not available in one patient.

Relation of histopathological fibrosis and inflammatory scores with mRSS

Patients with higher histological scores for the extent of dermal fibrosis had higher mRSS (rho 0.55, p=0.042) (Fig. 1). The fibrosis score was correlated with percentage of positive pixels in Masson's Trichrome staining (rho 0.78, p<0.001). Dermal inflammatory score did not correlate with the serum inflammatory markers C-reactive protein (CRP) or erythrocyte sedimentation rate (ESR). The correlation between dermal fibrosis and inflammatory score was 0.55 (p=0.008). CCN2 staining of fibroblasts correlated with the dermal inflammatory score (rho 0.58, p<0.001) and the histopathological fibrosis score (rho 0.44, p=0.041).

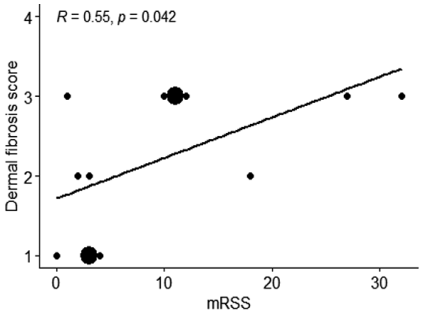

Fig. 1. The modified Rodnan skin score (mRSS) was higher in skin biopsies with severe fibrosis. The point size is increased with overlapping.

Cellular senescence

Senescence markers was more abundant in dermal fibroblasts in skin from SSc patients with elevated histopathological fibrosis score than in healthy control skin (Fig. 2). The correlation between fibrosis score and the score of senescence markers on fibroblasts was 0.43 for P16 (p=0.048) and 0.55 for P21 (p=0.008); the fibrosis score was also correlated with percentage of positive senescence pixels, including P16 (rho 0.49, p=0.022) and P21 (rho 0.44, p=0.040). There was hardly any Ki-67 co-localisation with senescence markers on fibroblasts or endothelial cells (Supplementary Fig. S1). In addition, increased senescence markers

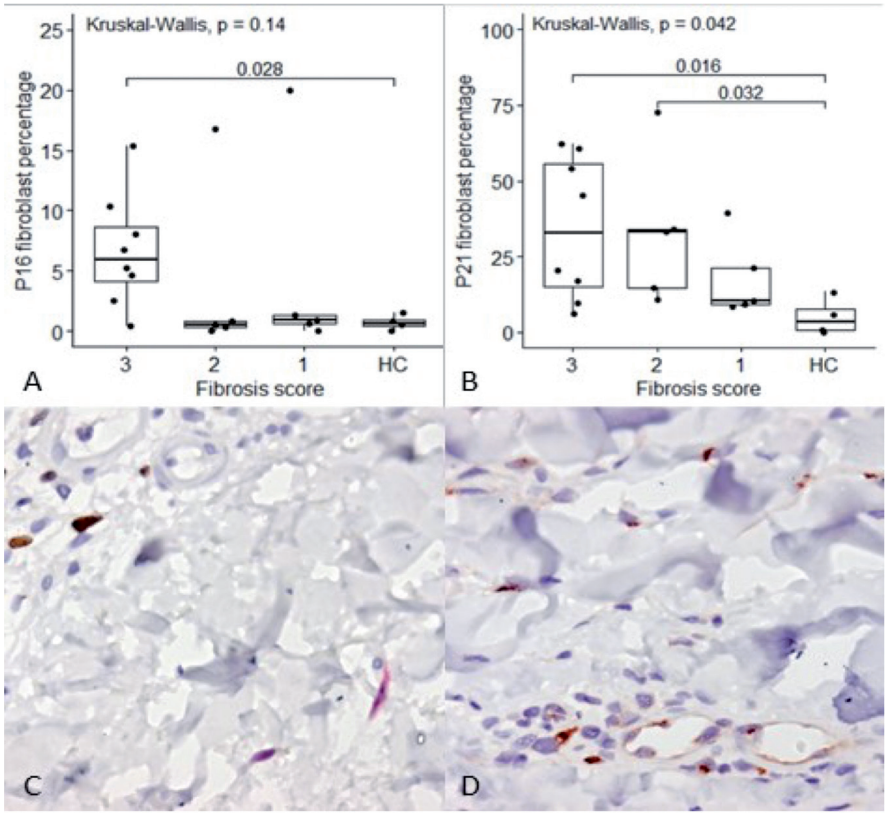

Fig. 2. Cellular senescence markers in fibrotic systemic sclerosis (SSc) skin biopsies.
A. P16 is increased in fibroblasts from the highly fibrotic dermis, but not in most of the milder cases.
B. P21 is more abundantly expressed than p16, and also increased in moderately fibrotic SSc biopsies.
C. P16 (bright red) expressed on fibroblasts without co-localisation of Ki-67 (brown). (D) CCN2 (brown) was expressed on fibroblasts and endothelium.

were associated with a higher CCN2-abundance on fibroblasts and increased dermal inflammatory score. The correlation between the abundance of CCN2 and senescence markers on fibroblasts was 0.63 for P16 (p<0.001) and 0.52 for P21 (p<0.001). The percentage of positive CCN2 pixels was also correlated with positive senescence pixels, including P16 (rho 0.54, p=0.010) and P21 (rho 0.44, p=0.042). The correlation between the dermal inflammatory score and senescence markers on fibroblasts was 0.45 for P16 (p=0.002) and 0.37 for P21 (p=0.014.) Moreover, there was only minimal abundance of senescence markers in endothelial cells, and this was not correlated with fibrosis severity (Supplementary Table S1).

Endothelial to mesenchymal transition

The frequency of EndMT was significantly higher in patients with SSc but did not differ between groups with different fibrosis severity according to mRSS and histopathological fibrosis score (Fig. 3A, B). The median (IQR) percentage of α-SMA- CD31 positive vessels was 6.4 (4.2-8.3) in SSc and 0.4 (0.0–1.4) in healthy controls (p=0.006). The median (IQR) percentage of α-SMA-ERG positive vessels was 9.1 (6.7-14.5) in SSc and 2.8 (1.7–3.9) in healthy control (p=0.003). Furthermore, the frequency of these EndMT features increased with the abundance of senescence markers and CCN2 on fibroblasts (Fig. 3C, D), as well as dermal inflammation. The percentage of α-SMA-ERG positive vessels was correlated with the percentage of CCN2 positive pixels (rho 0.52, p=0.014). The correlation between senescence and EndMT was observed in the percentage of α-SMA-ERG positive vessels and the percentage of P16 positive pixels (rho 0.53, p=0.010) as well as the percentage of α-SMA- CD31 positive vessels and the percentage of P21 positive pixels (rho 0.44, p=0.039). The median (IQR) percentage of CD31 and α-SMA co-localisation on vessels was 0.7 (0.0–3.5) in tissue free from inflammatory cells infiltration and 6.4 (4.0-8.4) in tissue with inflammatory cells infiltration (p=0.015). The median (IQR) percentage of vessels with enclosure of ERG positive endothelial cell nuclei by α-SMA stained cytoplasm was 9.8 (6.9–14.9) and 3.1 (2.1–3.5) in tissue with and without inflammatory cells infiltration, respectively (p<0.001). EndMT was not observed in lymphatic vessels (*i.e.* there was no co-localisation of D2-40 and α-SMA). The density of blood and lymphatic vessels did not correlate with inflammatory cell infiltration score, mRSS or histopathological fibrosis score.

Discussion

In this cross-sectional study of SSc skin biopsies, we observed more EndMT and fibroblast senescence in SSc skin biopsies compared to healthy control skin. The abundance of fibroblast senescence was also associated with the abundance of CCN2 on fibroblasts, and with the degree of fibrosis and inflammation in SSc skin. Our data reinforce the notion that EndMT and fibroblast senescence contribute to the pathogenesis of inflammation and fibrosis in SSc skin.

Senescence markers were increased in dermal fibroblasts from highly fibrotic SSc skin and were associated with CCN2. Senescent cells are in permanent cell cycle arrest and resist apoptosis (6). They undergo epigenetic changes resulting in altered, metabolically active phenotypes, including SASP, characterised by production and release of pro-fibrotic and pro-inflammatory cytokines (3). Studies with isolated dermal SSc fibroblasts have revealed SASP-like characteristics. They produced more mitochondrial reactive oxygen species (ROS) and displayed higher levels of phosphorylated TGF-β-activated kinase 1(TAK1) and downstream IKKβ–IRF5 signalling than healthy fibroblasts (11). The IRF5 pathway also regulated type 1 collagen α1, α2, MMP13, selectin P, selectin E, intercellular adhesion molecule 1, IL-6, PDGF-B and CCN2 in dermal fibroblasts (16). Although senolytic therapy with dasatinib, a tyrosine

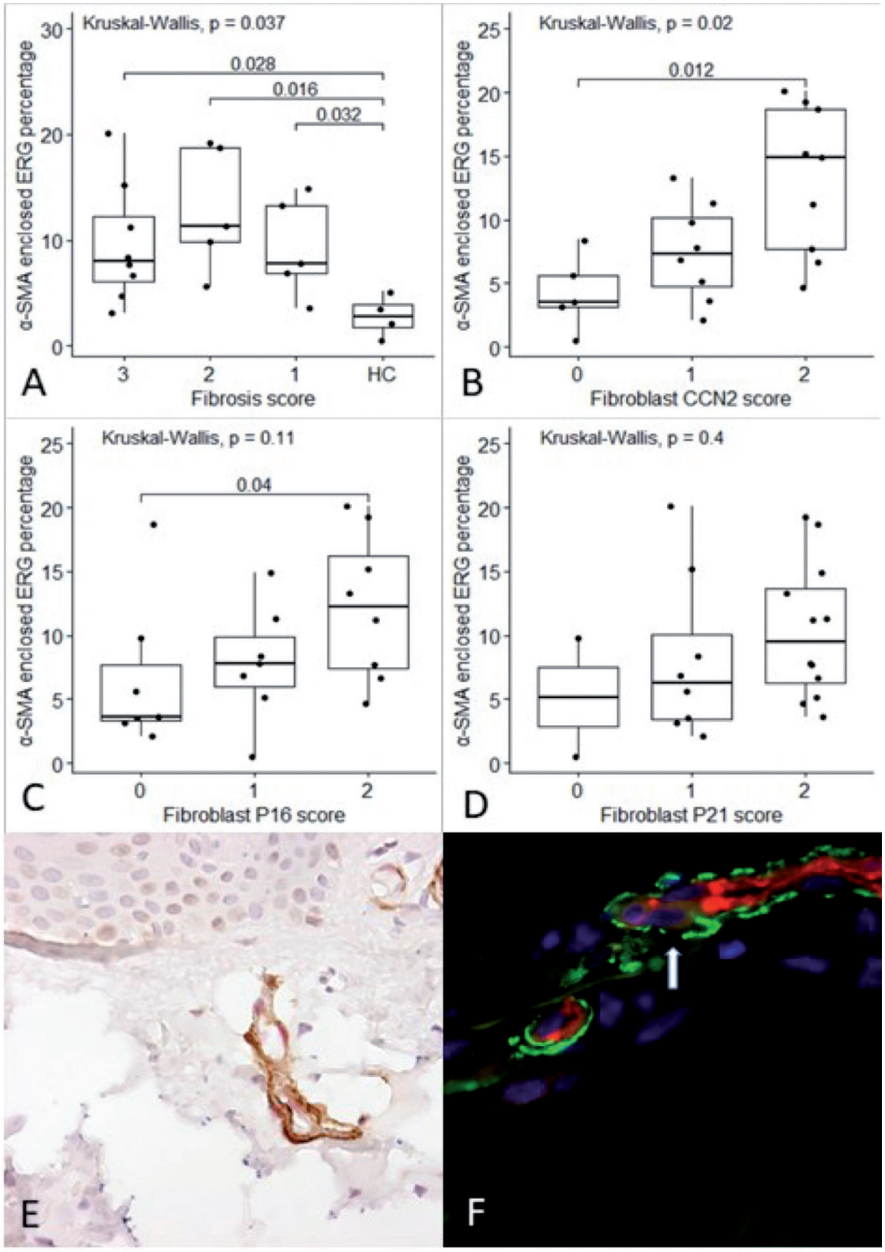

Fig. 3. A. Endothelial to mesenchymal transition (EndMT) was more prominent in systemic sclerosis (SSc) skin. Still, within the SSc group, there was no difference between biopsies with less or more severe fibrosis.
B, C, D. The frequency of EndMT increased with CCN2 and senescence markers on fibroblasts.
E. EndMT was detected by enclosure of ERG (bright red) positive endothelial cell nuclei by α-SMA (brown) stained cytoplasm under bright field.
F. EndMT was detected by co-localisation of CD31 (red) and α-SMA (green) immunofluorescent double staining.

kinase inhibitor, did not achieve a significant clinical effect on SSc-ILD and mRSS in an open-label phase 2a trial (17), the SSc patients with response to dasatinib showed a higher SASP signature of gene expression at baseline and a significant SASP expression reduction after senolytic therapy (3).

Our results also demonstrated that fibroblast senescence was associated with increased abundance of CCN2 and EndMT, possibly reflecting crosstalk between (senescent) fibroblasts and endothelial cells in SSc. TGF-β and CCN2 are not only iconic SASP but also capable of inducing EndMT, especially in response to oxidative stress in SSc (6-8). Senescent fibroblasts with SASP generate a paracrine effect on endothelia and aggravate fibrosis. In addition, fibroblasts have also been reported to interact with endothelial cells via NOTCH3 signalling in rheumatoid arthritis synovium, which accelerated IL-6 associated inflammation and fibroblast activation (18). The crosstalk between fibroblasts and endothelial cells thus points to a vicious circle of progressive fibrosis in SSc.

The mRSS was correlated with the dermal semi-quantitative fibrotic score. However, neither lymph nor blood vessel density correlated with dermal fibrosis in our study, while in another small study of SSc forearm skin biopsies, a decrease in dermal lymph density was noted (19). Moreover, EndMT was not observed on lymph vessels. Although both blood and lymph endothelial cells are capable of transdifferentiation to a more mesenchymal phenotype, the plasticity of remaining in a transition state between phenotypes may influence the detectability of EndMT in the snapshot of a skin biopsy (2, 20). Fully mesenchymal-transited endothelial cells would lose the typical cobblestone morphology, cell-cell junctions and EC markers, including CD31 and ERG, and therefore cannot be detected by double staining (21). Furthermore, CCN2 was also detected in fibroblasts and endothelium in healthy controls, especially in superficial dermis. Of note: CCN2 is not only involved in regulation of fibrosis but also in angiogenesis, chondrogenesis, and osteogenesis in soft tissue homeostasis (22). The production of CCN2 may be stimulated by pressure, sun light or chemicals exposure, especially in superficial dermis.

We utilised both digital analysis and semiquantitative scoring in this study. Digital analysis with colour deconvolution and pixel classification generates objective and reproducible data. On the other hand, semiquantitative scoring evaluates abundance of markers on specific target, including fibroblasts and vessels, which has yet to be satisfactorily proceed in digital analysis. In addition, confounding, including fat infiltration, glands, hair follicles, muscles, background staining and fissures, were relatively more manageable by experienced pathologists in semiquantitative scoring than

de-annotation in the software. An incorporation of both digital analysis and semiquantitative scoring connects the correlation of observed markers and possible origins.

Our study investigated histopathological features of skin biopsies taken for regular diagnostic purposes. Since skin biopsies are not an integral procedure for the diagnosis of SSc, our biopsies were derived from patients with apparently less complete or less typical clinical symptomatology. Despite the above, we only included patients meeting the ACR/EULAR 2013 classification criteria for SSc. Our study population was relatively heterogeneous because the biopsies were taken from various sites. Differences between normal skin characteristics in various areas of the body might have reduced the power of our analyses. For example, in sun-exposed skin, ultraviolet light can deregulate the TGF-β/SMAD pathway and induce cellular senescence (23, 24). Nevertheless, we found no significant difference of investigated markers between biopsy sites in our study; only for P16 and CCN2, there was a trend suggesting potentially higher abundance on dermal fibroblasts from upper limbs.

Conclusions

In summary, we observed EndMT and fibroblast senescence to be more abundant in SSc skin than in healthy control skin. Moreover, the abundance of fibroblast cellular senescence was associated with EndMT, dermal inflammation and fibrosis. Our data provide further support for the notion that fibroblast senescence and EndMT is implicated in the pathogenesis of skin fibrosis and inflammation in at least a subset of SSc patients. We propose that skin biopsies may serve as a valuable source for companion diagnostic biomarker assessment in further exploring (alternative) senolytics therapies in SSc.

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