# Overexpression of BAG3 (Bcl2-associated athanogene 3) in serum and skin of patients with systemic sclerosis

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## Abstract

### Objective

*BAG3 (Bcl2-associated athanogene3) is able to induce the transformation of cancer-associated fibroblasts to alpha smooth muscle actin (a-SMA) positive (+) myofibroblasts. In systemic sclerosis (SSc), a-SMA+ myofibroblasts also play an important role in the progression of fibrosis in the skin and involved internal organs. The aim of the study was to investigate whether BAG3 is overexpressed in SSc and may be a biomarker of fibrogenesis.*

### Methods

*BAG3 serum levels were measured in 106 patients with SSc, 47 with the limited (lc) and 59 the diffuse (dc) SSc, and in age- and sex-matched healthy controls (HC). BAG3 levels were then compared according to their clinical subset, nailfold video-capillaroscopic (NVC) patterns, interstitial lung disease (ILD, and correlated with modified Rodnan skin score (mRSS) and global disease activity. BAG3 expression was also investigated in skin biopsies of 8 dcSSc patients.*

### Results

*BAG3 serum levels were significantly higher in dcSSc (143.3 pg/mL, 95%CI 78–208.5) than in HC (0.68 pg/mL, 95%CI 0.13–1.23), and were significantly higher in patients with late NVC pattern and ILD but did not correlate with disease activity and mRSS. Of note, BAG3 was strongly expressed in the skin biopsies of dcSSc patients.*

### Conclusion

*BAG3 is overexpressed in dcSSc patients and may contribute to skin and organ fibrosis by prompting the transition of fibroblasts into myofibroblasts and increasing their survival. Thus, BAG3 may play an important role in SSc fibrotic pathogenesis and be a potential biomarker of fibrosis. Further research on its role as a therapeutic target is warranted.*

**Key words**

BAG3, systemic sclerosis, fibrosis, interstitial lung disease

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## Introduction

BAG3 (Bcl2-associated athanogene3) is a multifunctional protein that can bind to heat shock protein (Hsp) 70 through its BAG domain and to other partners through its WW, PXXP, IPV regions and 14-3-3 binding motifs (1, 2). It has been shown that its intracellular expression can be triggered by different stressful stimuli, such as oxidative stress and hypoxia. Intracellular BAG3 is constitutionally expressed in cells of skeletal muscle, heart, brain, and several tumours, and it has been shown that its activation is able to regulate many different pathways and functions, including apoptosis, autophagy, cytoskeleton organisation and motility (3).

A secreted form of BAG3 has been identified in pancreatic ductal adenocarcinoma and BAG3 gene overexpression has been found in other types of neoplasias (3). In pancreatic ductal adenocarcinoma, BAG3 induces the production of cytokines that supports the tumoural growth, by linking its specific receptor expressed in tumour-associated macrophages (4, 5). On the other hand, in cancer-associated fibroblasts, BAG3 regulates the expression of alpha smooth muscle actin (α-SMA) and the local recruitment of monocytes and, by a paracrine mechanism, induces enhanced intra-tumoural deposition of collagen matrix (6). The formation of such an inflammatory and fibrotic tumour microenvironment causes a more aggressive and drug-resistant phenotype of neoplasia (6).

Systemic sclerosis (SSc) is an autoimmune disorder in which the key feature is a chronic activation of the fibrotic process involving both the skin and internal organs (7). In this disorder, activated immune cells produce several cytokines, namely TGFβ, IL-6, FGF, and TNF-α, which are able to induce the transformation of resident fibroblasts to activated myofibroblasts characterised by intracellular expression of a-SMA and substantial deposition of collagen in the target organs and tissues (8). However, the biological pathways involved in activating and maintaining the fibrotic changes in SSc are still partially unknown.

Because of the correlation of BAG3 with fibrotic processes, in the present study we have investigated whether this protein may play some role in causing and maintaining the fibrotic process also in patients with SSc.

## Patients and methods

*Patients*

All the patients with SSc enrolled in this study met the ACR-EULAR classification criteria for this disorder (9). They were further classified as having the limited (lc) form or the diffuse (dc) form of SSc, according to the criteria of Le Roy *et al*. (10). A nailfold videocapillaroscopy (NVC) was performed in all the patients at the time of enrolment using a videocapillaroscope with a x 200 magnification lens. The derived digital images were stored using dedicated software (VideoCap; Scalar Co. Ltd., Tokyo, Japan). The observed capillaroscopic features were classified as early, active, and late patterns (11).

The presence of interstitial lung involvement (ILD) was assessed in all patients by spirometry and high-resolution chest computed tomography (HRCT). Based on the combined results of both spirometry and HRCT, patients were classified into 2 subgroups (Group 1 having a limited ILD and 2 having an extensive ILD, respectively), according to the criteria described by Goh *et al*. (12). In all patients, the level of disease activity was assessed by applying the EUSTAR activity index (13). Each patient was defined as having an active phase of the disease when this score was equal or more than 2.5. The modified Rodnan skin score (mRSS) (14), which is one of the items that make up the EUSTAR activity index, was evaluated at the time of enrolment, and also considered a statistical variable to be separately analysed.

Serum samples were obtained from all the patients and from 106 healthy controls (HC) and stored at -20°C for the subsequent analyses.

A skin biopsy was performed at a site where the skin involvement was clinically evident in 8 patients, all having the dcSSc variant.

A sex- and age-matched group of normal individuals was selected as a control group. A skin biopsy was obtained in 8 out of the normal volunteers at a site where the skin appeared normal.

*Ethical rules*

This study was conducted according to the Helsinki Declaration and approved by the local ethics committee (ID 3339, Study number 6549). Written informed consent was obtained from all of the enrolled patients, including those who underwent the skin biopsies.

*BAG3 sandwich ELISA test*

96-well microplates (MediSorp™ , cat. no. 467320, ThermoScientific, Waltham, MA, USA) were coated with 100 µl of solutions containing anti-BAG3 coating mAb (4 µg/ml in PBS 1X) and left overnight at 4°C. The day after, the wells were washed with PBS 1X, and the blocking of non-specific sites was performed for 2 hours at room temperature using PBS 1X containing 1% BSA (Merck KGaA, Darmstadt, Germany). When the blocking buffer was removed, 35 µl of BAG3 standard protein or 35 µl of serum samples was distributed in the appropriate wells with 35 µl of PBS 1X. Then 30 µl of adsorbent diluent (3.3% BSA+1.65 mg/ml bovine IgG+330µg/ml mouse IgG diluted in 0.165% tween 20–3.3X PBS) was added to each well. The plates were incubated at 37°C for 1 hour, then they were washed six times for 1 minute with the washing buffer, and 100 µl of Assay detector diluent (1% BSA+500 µg/ml bovine IgG+100 µg/ml mouse IgG+1µg/ml anti-BAG3 polyclonal HRP-conjugated antibody diluted in 0.05% tween 20–1X PBS) was loaded onto them. The plates were again incubated at 37°C for 30 minutes and then washed six times for 1 minute. Subsequently, TMB solution 1X (eBioscience, San Diego, CA, USA) was added to the wells and the colorimetric reaction was blocked by adding sulfuric acid 0.5 M. The optic density values (OD) were detected by the spectrophotometer at the wavelength of 450 nm.

*Immunofluorescence*

For paraffin-embedded sections, immunofluorescence protocol included deparaffination in Clear-Rite™ 3 (ThermoScientific, Waltham, MA, USA), rehydration through descending degrees of alcohol up to water, non-enzymatic antigen retrieval in sodium citrate buffer 10 mM, 0.05% Tween, pH 6.0, for 40 min in a pressure cooker at 95°C. After washing, non-specific binding was blocked with 10% normal goat serum (NGS) in PBS 1X 1h, RT. Sections were then incubated with an anti-PDGFRa (SAB4502142-100UG, Merck KGaA, Darmstadt, Germania; 1:200) and a murine anti-BAG3 mAb (3 µg/ml) overnight at 4°C in a humidified chamber. After another washing step, the sections were incubated with the secondary antibodies (DyLIGHT 488-conjugated AffinePure Goat anti-mouse and DyLIGHT 649-conjugated AffinePure Goat anti-Rabbit from Jackson ImmunoResearch, Cambridge, UK, used at 1:200 dilution). Nuclei were counterstained with 1µg/ml Hoechst 33342 (Molecular Probes, Eugene, Oregon, USA). The slides were then coverslipped using an aqueous mounting medium and analysed using a confocal laser scanning microscope (Leica SP5, Leica Microsystems, Wetzlar, Germany). The images were acquired in sequential scan mode using the same acquisition parameters (laser intensities, gain photomultipliers, pinhole aperture, x40 objective) when comparing experimental and control material. For the preparation of the figures, the brightness and contrast of the images were adjusted to leave a light cellular fluorescence background to visually appreciate the lowest fluorescence intensity features.

*Immunohistochemistry*

Five µm-thick sections of each tissue, mounted on poly-L-lysine-coated glass slides, were analysed by immunohistochemistry (IHC) using an anti-BAG3 mAb. IHC protocol included deparaffination in Clear-Rite™ 3, rehydration through descending degrees of alcohol up to water, incubation with 3% hydrogen peroxidase for 5 minutes to inactivate endogenous peroxidases, non-enzymatic antigen retrieval in EDTA at pH 8.0, for 30 minutes at 95°C. After rinsing with phosphate-buffered saline (PBS 1X), samples were blocked with 5% fetal bovine serum in 0.1% PBS/BSA and then incubated overnight at 4°C with the mAb in saturating conditions. The standard streptavidin-biotin linked horseradish peroxidase technique was then performed, and 3,3'-diaminobenzidine was used as a substrate chromogen solution for the development of peroxidase activity. Finally, the sections were counterstained with haematoxylin; slides were then cover-slipped using a synthetic mounting medium.

*Statistical analysis*

Statistical analysis of the collected data was performed using the MedCalc software package 2022 version (MedCalc® Inc., Ostend, Belgium) and Graphpad Prism software v. 9.5.1 (Boston, MA, USA). Spearman rank correlation was used to verify the presence of a relationship between BAG3 levels and the EUSTAR activity score and MRSS. Mann-Whitney non-parametric test was used to compare the levels of BAG3 in the normal population and in the different subset of patients subdivided according to their clinical classification (lcSSc and dcSSc, their NVC patterns and ILD extension). The unpaired t-test was used to compare the mean intensity of BAG3 between groups in the immunohistochemistry studies.

## Results

In this study, 106 patients with SSc and an equal number of age- and sex-matched normal controls were recruited. Of the patients with SSc, 47 were classified as having lcSSc and 59 dcSSc variants of the disease. Anti-centromere and anti-topoisomerase antibodies were present in 43 (40%) and 48 (45%) of the patients, respectively, while 14 patients (13%) showed high-titre positivity of anti-nuclear antibodies in the absence of antibodies that are considered specific for the disorder. The main clinical features of the patients with SSc, subdivided according the two different disease subsets, are detailed in Table I.

Serum levels of BAG3 were significantly higher in the SSc patients overall [mean value 85.3 pg/mL, 95% confidence interval (CI) 47.2–123.4] when compared with HC (0.68 pg/mL, 95% CI 0.13–1.23) ($p$=0.001). This finding can be totally ascribed to the very high levels of BAG3 found in patients with the dcSSc variant (143.3 pg/mL, 95%CI 78–208.5), whilst in patients with the lcSSc variant, the level is comparable to that observed in HC (12.58

pg/ml; 95%CI 5.34–19.82 *vs*. 25.41 pg/ml; 95% CI 15.45–35.36, respectively) (Fig. 1).

This result is also confirmed in all the skin biopsy sections obtained from the patients with dcSSc. A strong BAG3 overexpression is clearly demonstrated by both immune-histochemical and immunofluorescence methods in the biopsy sections, in comparison with the mild expression of the protein observed in the biopsies from normal subjects ($p$=0.0167) (Fig. 2). In addition, BAG3 appears to co-localise, at least in part, with the platelet-derived growth factor receptor a (PDGFRa).

BAG3 sera levels did not correlate with mRSS and with the EUSTAR activity score (Spearman $R^2$ = 0.344 and 0.203, respectively). Considering the different degrees of ILD observed in our dcSSc patient cohort, the levels of BAG3 are significantly higher in the patients with an extensive degree of ILD (mean value 243.60 pg/mL, 95% CI 134.10–353.00) in comparison with those with limited lung disease (mean value 24.43 pg/mL, 95% CI 3.06–45.80) ($p$=0.0005). (Fig. 3A). No correlation was found between BAG 3 serum levels and HRCT specific patterns.

In a similar way, the sera levels of BAG3 were significantly higher only in patients classified as having the late NVC pattern, which is indicative of a high degree of fibrotic changes in finger microcirculation (late NVC pattern mean value 190.20 pg/mL, 95% CI 101.3–279.1; early/active NVC pattern mean value 44.52 pg/mL, 95% CI 22.36–111.4) ($p$=0.0356) (Fig. 3B).

BAG3 values were not associated with any other disease domain including gastrointestinal involvement, as well as vascular features of the disease, *i.e*. digital ulcers, and pulmonary hypertension (data not shown).

**Table I.** Baseline demographic and clinical features of 106 patients with systemic sclerosis who were evaluated for BAG3 serum levels.

| Features | dcSSc (59) | lcSSc (47) |
|---|---|---|
| Age, median (range), years | 44 (20-70) | 46 (18-68) |
| Women, number (%) | 52 (88) | 44 (94) |
| Disease duration, median (range), years | 3.6 (0.6-7.2) | 5.2 (1-15) |
| mRSS | 6-24 | 2-8 |
| EUSTAR activity index, median (range) | 2.5 (0-9) | 0.55 (0-4) |
| FVC median (range), % of predicted value | 79 (54-131) | 100 (72-118) |
| Serum autoantibodies, n° (%) | | |
| Anti-nuclear antibody positive | 10 (16.9) | 4 (8.5) |
| Anti-topoisomerase positive | 48 (81.3) | 0 |
| Anti-centromere positive | 0 | 43 (91.4) |
| NVC patterns, | | |
| Early/Active | 20 | 40 |
| Late | 39 | 7 |
| Extension of ILD[§] | | |
| Limited disease | 31 | 41 |
| Extensive disease | 28 | 6 |

[§]According to the Goh score (11) (see text).

## Discussion

Our study clearly shows that BAG3 levels are strongly increased in the sera of patients with SSc, but this phenomenon appears to be limited to those suffering from the diffuse variant of the disorder.

This finding suggests that the pathologic pathways, whose activation is essential for the onset and progression of SSc, may be different in the limited and diffuse cutaneous variants of this disorder. Moreover, we show that BAG3 is overexpressed in the skin biopsies of dcSSc patients. This can be considered as an expected finding, considering that dermal tissue is the main target of the fibrotic process characterising the disease. In addition, BAG3 appears to co-localise with PDGFRα, which plays an important role in SSc-associated fibrosis. It has been clearly shown that a PDGF ligand abundance may strongly activate the PDGFR pathway inducing an enhanced production of fibrotic tissue in SSc (15).

Furthermore, higher levels of BAG3 appear to be strongly related to the latest stages of fibrotic evolution in distal finger microcirculation, analysed by NVC, and to the extension of ILD.

To our knowledge, this is the first demonstration of the possible participation of the BAG3 pathway to the pathological fibrotic process in patients with SSc.

This finding is in agreement with the correlation observed between BAG3 expression and the fibrosis extent in some type of tumours with a large stromal component, such as pancreatic ductal adenocarcinoma (4, 5). In this pathologic condition BAG3, directly secreted by tumour cells, is able to activate tumour-associated macrophages and induce them to produce some pro-tumour cytokines (4, 5). At the same time, BAG3 expression is correlated with the cancer-associated fibroblast (CAF) proliferation and differentiation to αSMA positive (+) myofibroblasts (6).

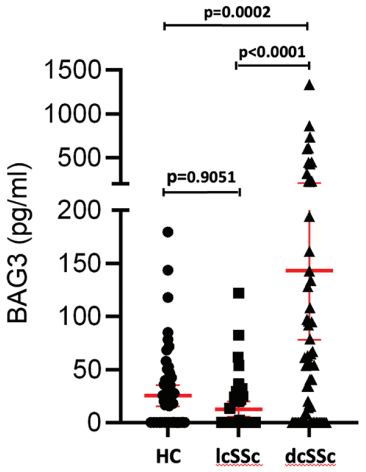


**Fig. 1.** BAG3 protein concentrations in serum samples from SSc patients.
Serum levels of BAG3 were significantly higher in dcSSc patients (143.3 pg/mL, 95% CI 78.0–208.5) with respect to those observed in lcSSc patients (12.6 pg/mL, 95% CI 5.3–19.8) ($p$<0.0001). It is remarkable that the BAG3 levels found in patients with lcSSc were not significantly different from those observed in NHS ($p$=0.9051).

The differentiation of reticular fibroblasts into collagen-producing α-SMA$^+$ myofibroblasts, by the action of biomechanical extracellular matrix (ECM) stiffness and biochemical factors such as transforming growth factor-β1 (TGFβ1) and connective tissue growth factor (CTGF), is a well-known mechanism of the fibrotic process in SSc, which is characterised by a substantial

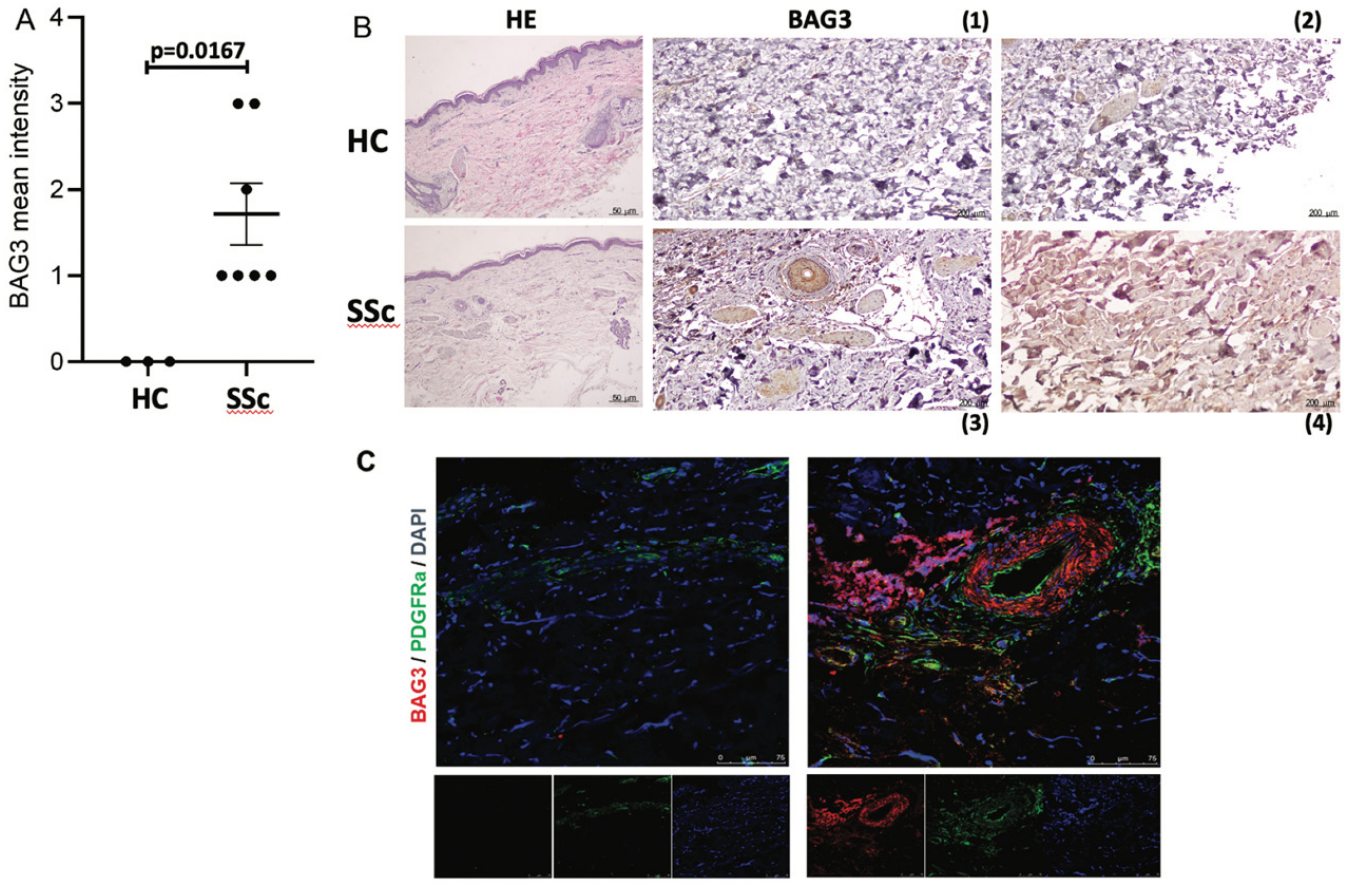


**Fig. 2.** BAG3 expression in skin biopsy of patients with dSSc and NHS.
Formalin-fixed and paraffin-embedded tissues were stained with an anti-BAG3 monoclonal antibody and scored based on the level of intensity and extent of staining.
**A**: BAG3 mean intensity between groups appears significantly different.
**B**: Representative haematoxylin and eosin (HE) and immunohistochemistry (IHC) images of BAG3 are shown (magnification: 2x and 10x). The mild expression of the protein observed in the biopsies taken from HC is displayed in fields B1 and B2. The fields B3 and B4 depict largely BAG3 positivity in the dermal vessels and fibroblasts.
**C**: PDGFRa$^+$ fibroblasts were identified in skin biopsy by immunofluorescence with an anti-PDGFRα antibody using confocal microscopy. The figure depicts the expression of BAG3 antigen (red) in PDGFRα (green)- positive cells.

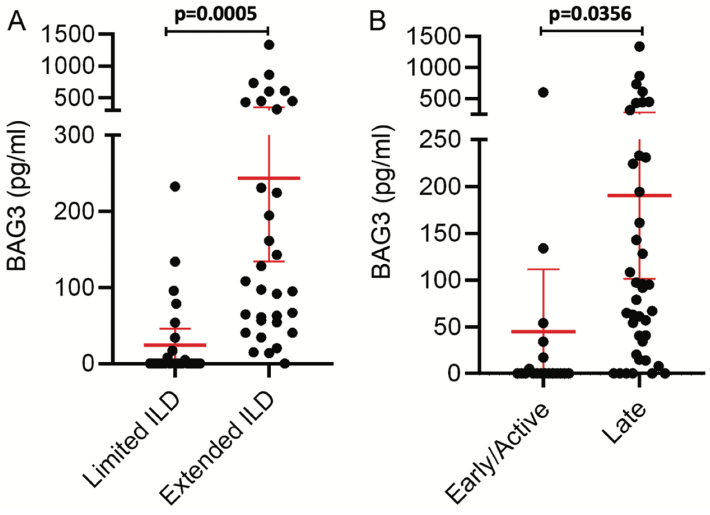


**Fig. 3.** BAG3 levels observed in dcSSc patients with different levels of ILD and different NVC patterns.
**A**: BAG3 values were significantly higher in patients with extended ILD (243.6 pg/ml; 95% CI 134.1-353.0) in comparison with limited ILD (n=27; 24.43 pg/ml; 95%CI 3.06–45.80) ($p$=0.0005).
**B**: BAG3 values were significantly higher in patients with the late NVC pattern (190.2 pg/ml; 95% CI 101.3–279.1) in comparison with early/active pattern (44.52 pg/ml; 95% CI 22.4–111.4) ($p$=0.0356).

production of collagen matrix in the skin tissue and in the stroma of internal organs such as lung, heart, and gastrointestinal tract (16). Activated myofibroblasts are important orchestrators in many physiological processes such as wound healing and tissue repair. Once myofibroblasts have completed their physiological function in these situations, they can die via apoptosis, a cell destiny induced by many apoptotic-inducing factors such as IL-1β, fibroblast growth factor 1 (FGF1) and prostaglandin E2 ($PGE_2$), or become senescent myofibroblasts, a cell type participating in ECM degradation (16). Alternatively, myofibroblasts and senescent myofibroblasts can escape apoptosis and continue to sustain a pathological fibrotic tissue production, as happens in fibrotic diseases like SSc (17). The

myofibroblast survival is a complex process due to different anti-apoptotic pathways. Two main pathways have been described. The first one is dependent on biochemical signalling of TGFβ1 which, linking to its membrane receptor, induces the production of anti-apoptotic factors such as BCL-XL and a series of BCL-2 anti-apoptotic proteins (17). The second pathway is triggered by biomechanical signalling derived from ECM stiffness, which induces the production of β1 integrin, with the consequent translocation into the nucleus of the transcriptional co-activator yes-associated protein (YAP) and transcriptional co-activator with a PDZ-binding motif (TAZ). These molecules are potent inductors of genes codifying for αSMA and TGFβ1. In this way the production of anti-apoptotic factors is further triggered (17, 18).

It is worth noting that BAG3 also possesses anti-apoptotic properties since its over-expression leads to the translocation into the nucleus of transcriptional co-activators YAP and TAZ. Thus, BAG3 may also act as a fibrosis inducer in SSc via its anti-apoptotic effect on myofibroblasts (19, 20).

In summary, our findings indicate that BAG3 may have an important pathological role in SSc by both inducing fibroblast differentiation to a-SMA+ myofibroblasts and favouring their survival by activating anti-apoptotic mechanisms. Other *in vivo* and *in vitro* studies are certainly needed to confirm the present data and better understand the effective contribution given by BAG3 to the development of chronic fibrotic processes in SSc.

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