Perivascular Cells in Diffuse Cutaneous Systemic Sclerosis Overexpress Activated ADAM12 and Are Involved in Myofibroblast Transdifferentiation and Development of Fibrosis

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ABSTRACT. Objective. Microvascular damage is pivotal in the pathogenesis of systemic sclerosis (SSc), preceding fibrosis, and whose trigger is not still fully understood. Perivascular progenitor cells, with profibrotic activity and function, are identified by the expression of the isoform 12 of ADAM (ADAM12) and this molecule may be upregulated by transforming growth factor-β (TGF-β). The goal of this work was to evaluate whether pericytes in the skin of patients with diffuse cutaneous SSc (dcSSc) expressed ADAM12, suggesting their potential contribution to the fibrotic process, and whether TGF-β might modulate this molecule.

Methods. After ethical approval, mesenchymal stem cells (MSC) and fibroblasts (FB) were isolated from bone marrow and skin samples collected from 20 patients with dcSSc. ADAM12 expression was investigated in the skin and in isolated MSC and FB treated with TGF-β by immunofluorescence, quantitative real-time PCR, and western blot. Further, we silenced ADAM12 expression in both dcSSc-MSC and -FB to confirm the TGF-β modulation.

Results. Pericytes and FB of dcSSc skin showed an increased expression of ADAM12 when compared with healthy control skin. TGF-β in vitro treatment induced a significant increase of ADAM12 in both SSc-MSC and -FB, with the higher levels observed in dcSSc cells. After ADAM12 silencing, the TGF-β ability to upregulate α-smooth muscle actin in both Ssc-MSC and Ssc-FB was inhibited.

Conclusion. Our results suggest that in Ssc, pericytes that transdifferentiate toward activated FB are present in the vascular tree, and TGF-β, while increasing ADAM12 expression, may modulate this transdifferentiation. (First Release June 1 2016; J Rheumatol 2016;43:1340–9; doi:10.3899/jrheum.150996)

Key Indexing Terms:
Systemic Sclerosis
Pericyte
Fibrosis

Fibrosis may be considered the hallmark of systemic sclerosis (SSc), affecting not only the skin but also different internal organs. In this setting, fibrosis represents the irreversible end stage triggered by different pathological events inducing...
During normal conditions, myofibroblasts, characterized by the presence of stress fibers containing α-smooth muscle actin (α-SMA), are involved in extracellular matrix (ECM) deposition and wound contraction during wound healing. Of note, when compared with normal skin, SSC skin shows a significant increase in myofibroblasts. The origin of activated myofibroblasts in the skin of patients with SSC is still a matter of debate. There is evidence that activated myofibroblasts may arise from local conversion of dermal FB and is also possible that resident and circulating mesenchymal progenitors may transdifferentiate toward activated myofibroblasts.

Finally, we demonstrated that SSC-endothelial cells (SSC-EC), under the synergistic effect of transforming growth factor-β (TGF-β) and endothelin 1, may further transdifferentiate toward myofibroblasts.

It has been suggested that pericytes and perivascular progenitor cells are involved in the process that, starting from chronic microvascular damage, evolves to fibrosis. In fact, lineage tracing experiments, in an experimental model of kidney fibrosis, confirmed that these cells are the main source of myofibroblasts, and the perivascular progenitor cells, with a profibrotic function, may be identified by the expression of 1 specific marker, the isoform 12 of ADAM (ADAM12). It is well known that the main expression of ADAM12 may be observed during embryonic morphogenesis of skeletal muscles and visceral organs, and intriguingly this molecule may be newly expressed in several human fibrotic diseases. Previously, we showed that mature pericytes obtained from patients with SSC, they are involved in the generation of myofibroblasts in this disease.

Translating the results obtained in experimental fibrosis, in our paper we assessed the expression of ADAM12 on the perivascular cells in the skin of patients with dcSSc and the ability of TGF-β, the pivotal profibrotic cytokine in SSC, to modulate this expression. The evidence of an increased ADAM12 expression on pericytes, positively modulated by TGF-β, supports the hypothesis that pericytes, which already transdifferentiate toward activated myofibroblasts, are present in the skin of patients with SSC and that these cells, mirroring other models of fibrosis, may contribute to the FB accumulation in the fibrotic skin of these patients.

MATERIALS AND METHODS

Patients, controls, and skin biopsies. Full-thickness biopsy samples, 2 × 0.5 cm, isolated from excisional biopsy, were obtained from clinically involved skin of one-third of the distal forearm of 20 patients with dcSSc according to LeRoy, et al. All patients fulfilled the 2013 American College of Rheumatology/European League Against Rheumatism classification criteria for SSC. Skin with a modified Rodnan skin score of ≥ 1 was considered clinically involved.

Of our patients, 50% were in a very early phase of SSC, considering that the term early, at present, refers to an undifferentiated connective tissue disease at higher risk to develop SSC, rather than a time at the beginning of the disease, as suggested by the pivotal study by Koenig, et al. We further divided our patients into 2 subsets: patients fulfilling the classification criteria in ≤ 1 year from the onset of Raynaud phenomenon [early-onset subset (EOS), n = 10], and all the others [longstanding subset (LSS), n = 10]. Skin samples from the same region of 10 age- and sex-matched healthy controls (HC) who underwent surgical treatment for trauma were used as controls. The skin samples were processed for immunofluorescence (IF) and FB cell isolation and culture. All patients with SSC underwent a 20-day washout from any immunosuppressive treatment and 1 month from intravenous prostanoids before performing skin biopsy. During this period, only proton-pump inhibitors and clebopride were allowed. Patients who could not undergo therapeutic washout because of severe organ complications were not enrolled in our study. Biopsies were taken after informed consent, and the study was approved by our local committee. Demographic and clinical characteristics of the patients are shown in Table 1. For IF, the specimens were fixed in 10% buffered formalin, dehydrated in graded alcohol series, and embedded in paraffin.

Immunofluorescence. The IF analysis was performed on paraffin sections (3-µm thickness) using an anti-ADAM12 antibody (Novus). Antigen retrieval was carried out using Target Retrieval Solution (Dako). The immunoreaction was revealed using a secondary antibody (Alexa fluore 488, Sigma-Aldrich). Negative controls were obtained by omitting the primary antibody. Vasculature pericytes were highlighted using a Cy3 conjugated mouse monoclonal anti-α-SMA antibody (Sigma-Aldrich) and an unconjugated anti-NG2 antibody (Santa Cruz, Biotechnology), EC using unconjugated anti-von Willebrand factor antibody (Dako), and FB using unconjugated anti-S100A4 antibody (Dako). The immunoreaction was revealed using a secondary antibody (Alexa fluor 555, Sigma-Aldrich). Cell nuclei were visualized using 4’, 6-diamidino-2-phenylindole. Fluorescence was analyzed using an Olympus BX53 fluorescence microscope. The intensity of fluorescence was measured using ImageJ software (National Institutes of Health (NIH)). The median cell count was evaluated by counting the number of S100A4+/ADAM12+ cells in 5 different high power fields (40x).

Isolation, culture, and immunophenotyping of MSC. After approval from the local ethics committee and written informed consent from patients, the bone marrow was obtained by aspiration from the posterior superior iliac crest from the patients enrolled in our study. Samples of MSC from bone marrow donors were used as controls.

MSC were obtained and expanded as previously described. Third-passage MSC were analyzed for the surface expression of MSC antigens (CD45, CD73, CD90, CD34, CD79a, PDGFR-β) and pericyte markers (α-SMA, SM22a, NG2, desmin, RG5), as previously described (data not shown).

FB isolation and culture. The skin specimen was placed into a 50-ml tube containing 10 ml of collagenase (Sigma) at 37°C for 2 h. After digestion, the samples were cultured in Dulbecco modified Eagle’s medium (Sigma) supplemented with 10% fetal bovine serum (FBS; Standard South America origin), 100 units/ml penicillin, and 100 ng/ml streptomycin (Sigma) at 37°C in a humidified atmosphere of 5% CO₂. The isolated cells were analyzed for the surface expression of S100A4 antigen by flow cytometry (FACSscan, Becton Dickinson) to assess their purity (S100A4+ cells > 99%).

MSC and FB treatment with TGF-β. The optimal concentration of TGF-β (R&D) was established with a dose/response curve evaluating ADAM12 mRNA expression in both MSC and FB after TGF-β administration for 7 days. The curve was performed using the MSC of 3 HC. Each experiment was performed in triplicate. The optimal stimulation dose for TGF-β was 10 ng/ml (data not shown). Both dcSSc and HC cells were cultured for 7 days in 1% FBS medium supplemented with optimal dose of TGF-β. Medium was changed every 2 days.

Small interfering RNA (siRNA) assay. dcSSc-MSC were seeded 24 h prior to transfection. At 70% confluence, the cells were transfected with ADAM12 siRNA (Life Technologies) or with Negative Control no-targeting scrambled Small interfering RNA (siRNA) assay.
siRNA (scr; Life Technologies) using Lipofectamine 2000 reagent (Life Technologies). MSC were transfected for 24 h with 50 pmol of siRNA in 2 ml of OptiMem. After incubation, cells were allowed to recover in normal growth conditions for 24 h post-transfection.

dcSSc-FB were transfected with the same siRNA construct used for MSC, and the transfection was performed using Lipofectamine 3000 (Life Technologies). Briefly, FB were plated 24 h prior to transfection. At 70% confluence, the cells were transfected for 24 h with 25 pmol of siRNA in 2 ml of OptiMem. After incubation, cells were allowed to recover in normal growth conditions for 24 h post-transfection. The expression of ADAM12 was determined by quantitative real-time PCR (qRT-PCR).

Western blot. MSC and FB, before and after TGF-β treatment, were pelleted, washed twice with phosphate buffered saline, lysed in lysis buffer (1% Triton X-100, 0.5% NP-40, 50 mM Tris-Cl, pH 7.5, 150 mM NaCl, 1 mM EDTA, supplemented with 1 mM phenylmethylsulfonyl fluoride, 1 mM NaF, 1 mM Na3VO4, 5 μg/ml aprotinin, 5µg/ml leupeptin) for 30 min and cleared by centrifugation. The protein concentration was calculated by Bradford protein assay reagent (Bio-Rad). Fifty micrograms of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel and transferred to nitrocellulose membranes.

After 1 h blocking at room temperature in blocking buffer (5% non-fat milk in Tris-buffered saline/1% Tween 20 (TBS/T)) and after washing 3 times for 5 min each in TBS/T, the membranes were incubated overnight at 4°C with the primary antibodies α-SMA (Abcam) and ADAM12 antibody (Novus) diluted in 5% bovine serum albumin in TBS/T. Following 3 washes with TBS/T, horseradish peroxidase-conjugated secondary antibodies (Santa Cruz Biotechnology) diluted in blocking buffer were added for 30 min at room temperature and washed 3 times with TBS/T. The detection was performed by enhanced chemiluminescence detection reaction (Amersham Pharmacia Biotechnology). All the signals were quantified by normalizing to the tubulin signal (CP06 Anti-α-Tubulin Mouse mAb-DM1A). Immunoreactive bands were quantified with densitometry using ImageJ software (NIH).

Results

ADAM12 expression in skin dcSSc. Figure 1 shows that ADAM12 expression in dcSSc skin was significantly higher when compared with HC skin. Further, in LSS dcSSc skin, the fluorescence intensity of ADAM12 expression in skin dcSSc.

RESULTS

ADAM12 expression in skin dcSSc. Figure 1 shows that ADAM12 expression in dcSSc skin was significantly higher when compared with HC skin. Further, in LSS dcSSc skin, the fluorescence intensity of ADAM12 expression evaluated using ImageJ was higher than that observed in EOS (Figure 1: B1–6, C1–6, and D).

ADAM12 was strongly expressed in pericytes, EC, and FB of dcSSc skin. A weak positivity of this molecule was observed in the same cells of HC skin.

In dcSSc skin, the number of SI100A4+/ADAM12+ FB surrounding the vessels was significantly higher than in HC skin.

Table 1. Clinical and demographic features of the 20 patients with dcSSc. The internal organ involvement refers to the time of the biopsies.

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<th>Autoantibodies</th>
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SSc: systemic sclerosis; dcSSc: diffuse cutaneous SSc; mRSS: modified Rodnan skin score (maximum possible score = 51); HRCT: high-resolution computed tomography; PFT: pulmonary function test; ANA: antinuclear antibodies; ScI-70: topoisomerase; PAH: pulmonary arterial hypertension.
Further, in the LSS dcSSc skin, the number of S100A4+/ADAM12+ FB surrounding the vessels (Figure 2, C1–2) was significantly higher when compared with EOS dcSSc skin (Figure 2, B1–2).

TGF-β induced an increased expression of ADAM12 in MSC and FB isolated by patients with dcSSc. Figure 3A shows that in untreated (UT) dcSSc-MSC, the mRNA levels of ADAM12 were significantly higher when compared with the levels of ADAM12 expression in UT HC-MSC [ADAM12 mRNA levels in UT MSC: 2.87 (2.27–3.95) in EOS dcSSc-MSC vs 0.98 (0.74–1.50) in HC-MSC, p < 0.0001, and 4.06 (2.65–4.34) in LSS dcSSc-MSC vs 0.98 (0.74–1.50) in HC-MSC, p < 0.0001]. After 10 ng/ml of TGF-β treatment for 7 days, we saw a significant increase in ADAM12, with the highest levels observed in dcSSc-MSC when compared with HC-MSC. [ADAM12 mRNA levels in TGF-β–treated MSC: 7.61 (6.23–9.56) in EOS dcSSc-MSC vs 3.42 (2.56–3.98) ADAM12 mRNA levels in HC-MSC, p < 0.0001, and 9.65 (8.36–10.43) in LSS dcSSc-MSC vs 3.42 (2.56–3.98) ADAM12 mRNA levels in HC-MSC, p < 0.0001]. No significant differences were observed in ADAM12 expression between EOS and LSS dcSSc-MSC.

The results obtained with perivascular MSC mirrored those observed in FB. The ADAM12 levels in UT HC-FB were in HC-MSC, p < 0.0001]. After 10 ng/ml of TGF-β treatment for 7 days, we saw a significant increase in ADAM12, with the highest levels observed in dcSSc-MSC when compared with HC-MSC. [ADAM12 mRNA levels in TGF-β–treated MSC: 7.61 (6.23–9.56) in EOS dcSSc-MSC vs 3.42 (2.56–3.98) ADAM12 mRNA levels in HC-MSC, p < 0.0001, and 9.65 (8.36–10.43) in LSS dcSSc-MSC vs 3.42 (2.56–3.98) ADAM12 mRNA levels in HC-MSC, p < 0.0001]. No significant differences were observed in ADAM12 expression between EOS and LSS dcSSc-MSC. The results obtained with perivascular MSC mirrored those observed in FB. The ADAM12 levels in UT HC-FB were lower than those in EOS and LSS dcSSc-FB.

Figure 1. ADAM12 expression in skin of patients with dcSSc. (A1–6) IF staining of HC skin. Microphotographs A1–3 show the same section stained with ADAM12 (green), vWF (red), and together. Microphotographs A4–6 show the same section stained with ADAM12 (green), α-SMA (red), and together. ADAM12 was strongly expressed in EC and pericytes of HC skin vessels. Microphotographs B1–3 show the same section stained with ADAM12 (green), vWF (red), and together. Microphotographs C4–6 show the same section stained with ADAM12 (green), α-SMA (red), and together. ADAM12 was strongly expressed in EC and pericytes of EOS dcSSc skin vessels. (C1–6) IF staining of LSS dcSSc skin. Microphotographs C1–3 show the same section stained with ADAM12 (green), vWF (red), and together. Microphotographs C4–6 show the same section stained with ADAM12 (green), α-SMA (red), and together. ADAM12 was strongly expressed in EC and pericytes of LSS dcSSc skin vessels. (E1–3) IF staining of EOS dcSSc skin. Microphotographs show the same section stained with ADAM12 (green), NG2 (red), and together. ADAM12 was strongly expressed in EC and pericytes of EOS dcSSc skin vessels. (F1–3) IF staining of LSS dcSSc skin. Microphotographs show the same section stained with ADAM12 (green), NG2 (red), and together. ADAM12 was strongly expressed in EC and pericytes of LSS dcSSc skin vessels. (G) Densitometric analysis of the IF intensity for ADAM12. Results are expressed as median (range) of the IF intensity measured using ImageJ. A significant increase of ADAM12 expression was observed in patients with dcSSc when compared with HC. HC vs EOS dcSSc: ** p = 0.0002. HC vs LSS dcSSc: *** p = 0.0001. EOS dcSSc vs LSS dcSSc: ** p = 0.0002. dcSSc: diffuse cutaneous systemic sclerosis; IF: immunofluorescence; vWF: von Willebrand factor; EC: endothelial cells; HC: healthy controls; EOS: early-onset subset; LSS: longstanding subset; α-SMA: α-smooth muscle actin.

Figure 2. ADAM12 expression in perivascular FB of dcSSc skin. (A–C) IF staining of HC skin (A1–2), EOS dcSSc skin (B1–2), and LSS dcSSc skin (C1–2). The microphotographs A1, B1, and C1 show the merger of double-staining of ADAM12 (green) and vWF (red) at 10× magnification. The areas inside the squares are shown in microphotographs A2, B2, and C2 at 40× in the consecutive section and stained with ADAM12 (green) and S100A4 (red). The ADAM12+ perivascular cells coexpresses the FB marker S100A4. (D) Median number of S100A4+ADAM12+ cells obtained in 5 different HPF. The number of S100A4+ADAM12+ cells was significantly higher in LSS dcSSc skin when compared to EOS dcSSc skin. Any dot plot is representative of the median cells count per 5 HPF (40x) for each patient. HC vs EOS dcSSc: ** p = 0.0002. HC vs LSS dcSSc: ** p = 0.0002. EOS dcSSc vs LSS dcSSc: ** p = 0.0002. FB: fibroblasts; dcSSc: diffuse cutaneous systemic sclerosis; IF: immunofluorescence; HC: healthy controls; EOS: early-onset subset; LSS: longstanding subset; vWF: von Willebrand factor; HPF: high power fields.
Figure 3. TGF-β induced an increased expression of ADAM12 in MSC and FB isolated by patients with dcSSc. (A–B) The quantification by qRT-PCR of ADAM12 mRNA levels in (A) MSC and (B) FB. The treatment with TGF-β induced a significant increase of ADAM12 when compared with UT in both HC-MSC and dcSSc-MSC. The value of ADAM12 mRNA expression was a significant increase in dcSSc-MSC without a difference between EOS and LSS patients. Any single dot in the figures represents the median of triplicate experiments for each patient. ** p = 0.0002. *** p = 0.0001. (C–D) Western blot analyses confirmed the results observed by qRT-PCR analyses. Pictures are representative of all experiments. Protein bands were quantified by densitometry and the values were expressed as protein relative quantification/α-tubulin relative quantification. * p = 0.002. ** p = 0.0002. *** p = 0.0001. (E) IF staining of ADAM12 in dcSSc-MSC and HC-MSC. ADAM12 localization in UT dcSSc-MSC is widely diffuse in the cytoplasm, suggesting its activation state, and displays the same pattern observed in HC-MSC treated with TGF-β. Pictures are representative of all experiments. Original magnification 20x. (F) IF staining of ADAM12 in dcSSc-FB and HC-FB. Mirroring the results observed in MSC, ADAM12 localization in UT dcSSc-FB is widely diffuse in the cytoplasm and displays the same pattern observed in HC-FB treated with TGF-β. Pictures are representative of all experiments. Original magnification 20x. TGF-β: transforming growth factor-β; MSC: mesenchymal stem cells; FB: fibroblasts; dcSSc: diffuse cutaneous systemic sclerosis; IF: immunofluorescence; qRT-PCR: quantitative real-time PCR; UT: untreated cells; HC: healthy controls; EOS: early-onset subset; LSS: longstanding subset.

TGF-β treatment induced a significant upregulation of (Figures 4C and 4D). Our results show that pericytes of patients with SSc express expression in the same cells (Figures 4E, 4F, and 4G).

DISCUSSION

Our results confirm the presence of ADAM12+ cells in the perivascular areas of the affected skin of patients with dcSSc, and the expression of S100A4+/ADAM12+ cells in the fibrotic skin was limited to close to the vessels in EOS patients. Further, we showed an increased ADAM12 fluorescence intensity in LSS patients when compared with EOS patients, and this enhanced intensity was associated with an increased number of S100A4+/ADAM12+ cells and with the severity of the fibrosis. In addition, these cells in LSS patients were widely distributed in the fibrotic area.

In a mouse treated with bleomycin, an experimental model of SSC, it has been shown that pericytes may contribute to skin fibrosis. Further, we provided evidence of a profibrotic phenotype of human perivascular cells expressing increased levels of α-SMA and collagen during SSC. It may modulate the production of profibrotic molecules in perivascular dcSSc-MSC, thus eliciting a profibrotic phenotype. The findings of ADAM12 hyperexpression and activation in SSC perivascular cells support the hypothesis of the profibrotic involvement of these cells during SSC.
Taken together, these data lead us to speculate that perivascular cells of patients with dcSSc undergoing myofibroblast differentiation move from the perivascular areas to colonize the affected tissues and contribute to the FB recruitment and accumulation.

The functional involvement of ADAM12 in myofibroblast generation during SSC is still a matter of debate. ADAM12 plays a direct profibrotic role, regulating TGF-β signaling\(^{23,42,43}\). After TGF-β binds with specific receptors, the signal is transduced to the nucleus by members of the Smad and Mad-related (Smad) family. It has been reported\(^{23,44}\) that, in vitro, TGF-β stimulation induces an accumulation of ADAM12, in turn inducing Smad phosphorylation. On the other hand, depletion of endogenous ADAM12 by siRNA suppresses Smad phosphorylation\(^{44}\). In our setting, the levels of ADAM12 were significantly higher in SSc cells when compared with HC cells, already before the TGF-β stimulation. After TGF-β treatment, a significant increase of ADAM12 was shown in both SSc and HC cells, the highest levels observed in the cells of patients with SSc. We may suggest that, during SSc, a pathological environment enriched in TGF-β may contribute to pericytes differentiation toward...
myofibroblasts through ADAM12 upregulation, which acts as a positive regulator of profibrotic TGF-β signaling.

The inactive form of ADAM12 is normally located in the perinuclear region of the cell. After TGF-β stimulation, ADAM12 loses its prodomain, migrating into the cytoplasm as a mature molecule where it is catalytically active. In our experiments, we showed that in both HC-MSC and HC-FB, the TGF-β treatment induced an intracellular redistribution of ADAM12 from the perinuclear region to the cytoplasm. But in both dcSSc-MSC and dcSSc-FB, ADAM12 was already located in the cytoplasm, suggesting that during SSC, the pathological environment, enriched in TGF-β, may increase the active form of ADAM12.

In this activated status, ADAM12 may be involved in the transdifferentiation of both MSC and FB toward myofibroblasts, as shown by the increased α-SMA expression. To confirm the functional role of ADAM12 in α-SMA induction, after ADAM12 silencing and in vitro TGF-β stimulation, we showed that the ADAM12-silenced cells were unable to induce α-SMA expression. Further, after ADAM12 silencing, the SSc-FB were unresponsive to TGF-β stimulation and were confirmed to induce Col1A1, α-SMA, and CTGF gene expression.

Our results showed that not only pericytes, but also dcSSc-EC of the dermal vessel expressed ADAM12. The involvement of this molecule in EC is still not fully understood. During the physiological angiogenesis, many metalloproteinases are responsible for ECM degradation to support EC invasion. Pathological activation of ADAM12 seems to be associated with the dissolution of adherent junctions and the loss of cell-cell contacts, resulting in EC apoptosis, as reported by an in vitro study performed on EC. On these bases, we speculate that the increase of ADAM12 expression in a dysfunctional endothelium may be associated with the loss of cell-cell contacts, resulting in vessel rarefaction and avascular areas, as observed in SSC.

Several studies focused on pericytes as a possible source of the activated myofibroblasts observed during different fibrotic conditions, including SSC. In our work, we provide evidence of activated ADAM12 expression in SSc perivascular cells, suggesting their tendency toward profibrotic activity. Further, the evidence of S100A4+/ADAM12+ cells located in the perivascular areas during the early phase of the disease and widely diffused in the fibrotic areas during longstanding disease leads us to hypothesize their possible origin from perivascular cells that successively migrated into the affected tissues.

Further studies analyzing the relationship between ADAM12 and ALK5, or analyzing the inhibition of ADAM12 functions, may support the hypothesis that targeting ADAM12 can prevent fibrosis, a clinical pattern still needing effective therapies.

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