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Original article

Inorganic pyrophosphate is reduced in patients with systemic sclerosis

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Abstract

Objective. The pathogenesis of calcinosis cutis, a disabling complication of SSc, is poorly understood and effective treatments are lacking. Inorganic pyrophosphate (PPi) is a key regulator of ectopic mineralization, and its deficiency has been implicated in ectopic mineralization disorders. We therefore sought to test the hypothesis that SSc may be associated with reduced circulating PPi, which might play a pathogenic role in calcinosis cutis.

Methods. Subjects with SSc and age-matched controls without SSc were recruited from the outpatient rheumatology clinics at Rutgers and Northwestern Universities (US cohort), and from the Universities of Szeged and Debrecen (Hungarian cohort). Calcinosis cutis was confirmed by direct palpation, by imaging or both. Plasma PPi levels were determined in platelet-free plasma using ATP sulfurylase to convert PPi into ATP in the presence of excess adenosine 5' phosphosulfate.

Results. Eighty-one patients with SSc (40 diffuse cutaneous, and 41 limited cutaneous SSc) in the US cohort and 45 patients with SSc (19 diffuse cutaneous and 26 limited cutaneous SSc) in the Hungarian cohort were enrolled. Calcinosis was frequently detected (40% of US and 46% of the Hungarian cohort). Plasma PPi levels were significantly reduced in both SSc cohorts with and without calcinosis (US: $P = 0.003$; Hungarian: $P < 0.001$).

Conclusions. Circulating PPi are significantly reduced in SSc patients with or without calcinosis. Reduced PPi may be important in the pathophysiology of calcinosis and contribute to tissue damage with chronic SSc. Administering PPi may be a therapeutic strategy and larger clinical studies are planned to confirm our findings.

Key words: SSc, calcinosis, ectopic mineralization, inorganic pyrophosphate, hydroxyapatite

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Rheumatology key messages

- Circulating inorganic pyrophosphate binds directly to hydroxyapatite crystal to inhibit its growth.
- We found reduced circulating inorganic pyrophosphate levels in scleroderma patients with or without calcinosis.
- This may contribute to pathophysiology of calcinosis and administering inorganic pyrophosphate may be a therapeutic strategy.

Introduction

Ectopic mineralization, characterized by the deposition of calcium/phosphate complexes in connective tissues in aberrant locations, complicates a wide variety of diseases, as well as ageing. Ectopic mineralization has been linked to cancer, diabetes and rheumatic autoimmune diseases, and contributes to morbidity and mortality in these conditions [1, 2]. Two major types of acquired ectopic mineralization processes involving peripheral connective tissues have been recognized. Metastatic mineralization refers to calcium deposition in the skin and vascular walls associated with elevated serum levels of phosphate and/or calcium, as in chronic renal failure and calciphylaxis. In contrast, dystrophic mineralization is usually secondary to tissue injury, as seen in cancer, ageing and autoimmune systemic diseases such as SSc, SLE and inflammatory myopathy [3–5]. In osteoarthritis, dystrophic mineralization of the ageing cartilage occurs with deposition of basic calcium phosphate crystals, which is believed to play a pathogenic role in the cartilage degradation process [6–8]. Similarly, the destructive process of Milwaukee Shoulder Syndrome is attributed to these basic calcium phosphate deposits in the shoulder joint. Basic calcium phosphate deposits may also be found in peri-articular structures. However, in the setting of osteoarthritis, these crystals do not deposit in the soft subcutaneous connective tissues, which occurs in the autoimmune systemic rheumatic diseases. This dystrophic calcinosis can be widespread and is thought to result from multiple contributing metabolic and environmental factors that obscure efforts to uncover the precise basis of these disorders [9]. Up to 40% of SSc patients [3], 44–70% of patients with juvenile dermatomyositis [4] and 10–20% with adult dermatomyositis [4, 5] will develop calcinosis. These painful soft tissue deposits may enlarge over time and contribute to disability, including pain, infections and joint contractures. There is currently no effective medical treatment for calcinosis cutis or strategies to prevent established deposits from enlarging [10]. Importantly, not all patients with SSc develop calcinosis. However, there are no markers to predict who will develop this painful complication.

Several Mendelian genetic disorders share phenotypic similarities with the acquired forms of metastatic and dystrophic mineralization and serve as genetically controlled model systems to study pathological mineralization [11–15]. Pseudoxanthoma elasticum (PXE) is a prime example of heritable connective tissue disorders associated with prominent ectopic mineralization in the skin, eyes and wall of arterial blood vessels. In contrast to the late-onset and slowly progressive development of the ectopic mineralization in PXE, generalized arterial calcification of infancy is a severe disease often diagnosed by prenatal ultrasound. In the spectrum of heritable ectopic mineralization disorders, arterial calcification due to deficiency of CD73 (ACDC) is an adult-onset disorder with ectopic mineralization in arteries of the lower extremities, and peri-articular ligaments in elderly individuals. PXE, generalized arterial calcification of infancy and ACDC are caused by loss-of-function mutations in the *ABCC6*, *ENPP1* and *NT5E* genes, respectively. Recent studies in murine models and patients with these diseases indicated that all three conditions are associated with reduced plasma levels of inorganic pyrophosphate (PPi) [13, 15]. PPi was first identified as the most potent endogenous inhibitor in biomineralization in the 1960s [16]. PPi acts by binding to the surface of hydroxyapatite (calcium phosphate) crystals and directly inhibits hydroxyapatite crystal growth [17]. Plasma PPi levels have ***not*** been studied in the rheumatic diseases prone to tissue calcification such as SSc.

In this study, we sought to examine plasma PPi levels in patients with SSc [18] with and without calcinosis, and compared with a control population without SSc, including rheumatic diseases not associated with calcinosis, such as RA [19], fibromyalgia [20] and osteoarthritis [21].

Methods

Patient recruitment

Upon IRB approval (Institutional Review Board of Rutgers-Robert Wood Johnson Medical School, New Brunswick, New Jersey (Pro20170001960)

—Institutional Review Board of Northwestern University, Chicago, Illinois (# STU00208246)), subjects meeting 2013 ACR criteria for SSc [18] and age-matched controls without SSc were recruited from two United States (US) cohorts: Rutgers-RWJ and Northwestern Rheumatology outpatient clinics and compared with a similar cohort from Hungary: Department of Rheumatology and Immunology, University of Szeged, and the Division of Rheumatology, University of Debrecen. The study protocol was approved by the

Ethics Committee of the Hungarian National Public Health Centre (No: 16985–9/2020). Each subject provided written informed consent to participate in this study. Patients with scleroderma had either known calcinosis or never diagnosed (either by history, physical exam or imaging) with calcinosis. These were compared with a control group (either with rheumatic disease but without SSc or no rheumatic disease) who had diagnoses not known to be associated with dystrophic mineralization of the soft subcutaneous tissues, including inflammatory arthritis [19], fibromyalgia [20] and osteoarthritis [21]. Calcinosis was confirmed by the following: a previous history of drainage from these sites, or surgical removal of symptomatic deposits, by direct palpation on physical exam, or imaging of the affected area(s). Subjects were separated into different subgroups: subjects with calcinosis *vs* subjects without calcinosis; furthermore, SSc subjects were separated into those with diffuse cutaneous SSc *vs* limited cutaneous SSc and compared with control population without SSc. Demographic and clinical history were obtained from electronic medical records, including pertinent diagnosis of osteoporosis, parathyroid and thyroid disease, diabetes, cancer, medications and laboratory studies, including electrolytes and renal functions. Pertinent data regarding the primary rheumatic diagnosis were also collected when available, including the type of disease, disease duration and auto-antibodies. For the SSc patients, additional data on disease duration (from non-Raynaud symptoms), autoantibody profile, the most important SSc-related organ manifestations (e.g. interstitial lung disease: defined as abnormal restrictive pattern on full pulmonary function tests and confirmed by high resolution chest CT scan of the lungs; pulmonary arterial hypertension: confirmed by right heart catheterization; history of digital ulceration: defined as loss of epithelialization; the degree based on the depth into the layers of the skin affected, including the epidermis, dermis and subcutaneous tissues of the digital tip) and types of immunosuppressive and vascular therapy were also recorded.

PPi assays

Whole blood was collected into CTAD blood collection tubes and transferred immediately to EDTA tubes (US cohort) or without EDTA (Hungarian cohort). Plasma was collected after centrifugation by 2000 *g* for 15 min at 4°C. To deplete platelets, a rich source of PPi that could interfere with the assay, plasma was filtered by 2200 *g* for 30 min at 4°C through a Centrisart I 300 kDa mass cut-off filtration unit (Sartorius, New York, NY, USA) and stored at −80°C until analysis [19]. Circulating PPi in plasma was measured by an enzymatic reaction using ATP sulfurylase (ProSpec, Ness-Ziona, Israel) to convert PPi into ATP in the presence of excess adenosine 5' phosphosulfate (Sigma-Aldrich, St Louis, MO, USA), (the ATPS assay) as described previously [22, 23]. Additionally, circulating PPi in platelet-free plasma was also measured by a separate enzymatic assay in the US cohort, using ^{14}C labelled uridine-diphosphoglucose pyrophosphorylase [24–27]. All specimens in the US cohort were performed by Q.L. at Jefferson University and by E.K. and L.B.'s labs in the Hungarian cohort. The ATPS assay protocol used in the US and Hungarian cohorts was performed exactly the same way at both sites, because the investigators at these sites have collaborated extensively on PXE and generalized arterial calcification of infancy genetic disorders [23, 28].

Statistical analysis

Demographics and PPi results were compared between the control, diffuse SSc and limited SSc groups using analysis of variance. Demographics, duration of disease and PPi results were compared between the diffuse SSc and limited SSc groups using two-way *t*-tests. PPi results were compared by SSc and calcinosis status using an analysis of variance. Quartiles were calculated with an outlier cut-off of 1.5 times the interquartile range. All statistical analysis was performed with Stata Version 14 (StatCorp).

Results

Table 1 shows that the US cohort comprised of 81 SSc and 15 control subjects, and the Hungarian cohort consisted of 45 SSc and 26 control subjects. The US control population consisted of one patient with stable SLE, seven with inflammatory arthritis and seven with osteoarthritis. The Hungarian control population were healthy subjects without rheumatic disease. Both SSc cohorts were largely comparable and more than three-quarters were females; the Hungarian SSc subjects were older. Both cohorts with limited cutaneous SSc had significant longer disease duration compared with the diffuse cutaneous SSc population. Calcinosis was present in 40.7% of the US SSc cohort and 46.7% of the Hungarian cohort. More than 90% of SSc-calcinosis subjects had hand involvement, which was confirmed by physical exam or imaging, or both. Those with more proximal or truncal involvement who were symptomatic usually had imaging to assess the extent of their deposits. Circulating PPi levels were significantly diminished in both SSc cohorts using the ATPS method and the difference was even more robust using the C14 assay in the US cohort ($P < 0.001$) (Supplementary Table S1, available at *Rheumatology* online). Supplementary Tables S2 and S3, available at *Rheumatology* online, showed more significant use of immunosuppression, corticosteroid and osteoporosis therapy in the US population with diffuse SSc; similarly, the Hungarian cohort with diffuse SSc also received significantly more immunosuppressive therapy, and their limited SSc cohort had significantly more thyroid disease. Supplementary Tables S4 and S5, available at *Rheumatology* online, showed similar SSc clinical characteristics between those with and without calcinosis in both cohorts.

Table 1 Demographics and PPi levels in control (no SSc) and SSc cohorts

	No SSc	SSc – diffuse cutaneous	SSc – limited cutaneous	Total	*P*-Anova
US cohorts					
n (%)	15 (15.6)	40 (41.7)	41 (42.7)	96 (100.0)	
Female, *n* (%)	12 (80.0)	33 (82.5)	34 (82.9)	79 (82.3)	0.967
Age, mean (S.D.), years	57.2 (17.3)	54.1 (12.4)	56.5 (13.1)	55.6 (13.5)	0.636
Calcinosis, *n* (%)		16 (40.0)	17 (41.5)	33 (40.7)	0.893[b]
Disease duration,[a] mean (S.D.), years		8.9 (8.4)	12.8 (8.0)	10.9 (8.4)	**0.036**[b]
PPi ATPS assay, mean (S.D.)	1.2 (0.3)[A]	1.0 (0.2)[B]	1.1 (0.2)[A]	1.1 (0.2)	**0.003**
Hungarian cohort					
n (%)	26 (36.6)	19 (26.8)	26 (36.6)	71 (100.0)	
Female, *n* (%)	23 (88.5)	15 (78.9)	25 (96.2)	63 (88.7)	0.197
Age, mean (S.D.), years	61.9 (10.8)	58.3 (12.5)	61.5 (10.2)	60.8 (11.0)	0.515
Calcinosis, *n* (%)		10 (52.6)	11 (42.3)	21 (46.7)	0.493[b]
Disease duration,[a] mean (S.D.), years		8.1 (6.0)	13.5 (8.4)	11.2 (7.9)	**0.022**[b]
PPi ATPS assay, mean (S.D.)	1.7 (0.3)[A]	1.4 (0.3)[B]	1.4 (0.4)[B]	1.5 (0.3)	**<0.001**

Bold indicates statistically significant at the $P < 0.05$ level. Different superscript upper-case letters indicate groups are statically significantly different by Tukey's HSD at the $P < 0.05$ level. PPi: inorganic pyrophosphate. [a]Years from onset of non-Raynaud's symptoms. [b]*P*-value from *t* test.

Fig. 1 shows statistically lower plasma PPi levels in the diffuse compared with limited SSc in the US cohort ($P = 0.021$) but no difference in the Hungarian cohort ($P = 0.942$). However, Fig. 2 shows that plasma PPi levels were not statistically different between SSc patients with or without calcinosis (US cohort $P = 0.161$, Hungarian cohort 0.665).

Discussion

To our knowledge, plasma PPi levels have not been studied in the rheumatic diseases prone to tissue calcification such as SSc. Herein, we show that circulating PPi levels are reduced (by ~20%) in patients with SSc compared with an age-matched control population

Fig. 1 Distribution of PPi values by type of SSc for the US (left) and Hungarian (right) cohorts

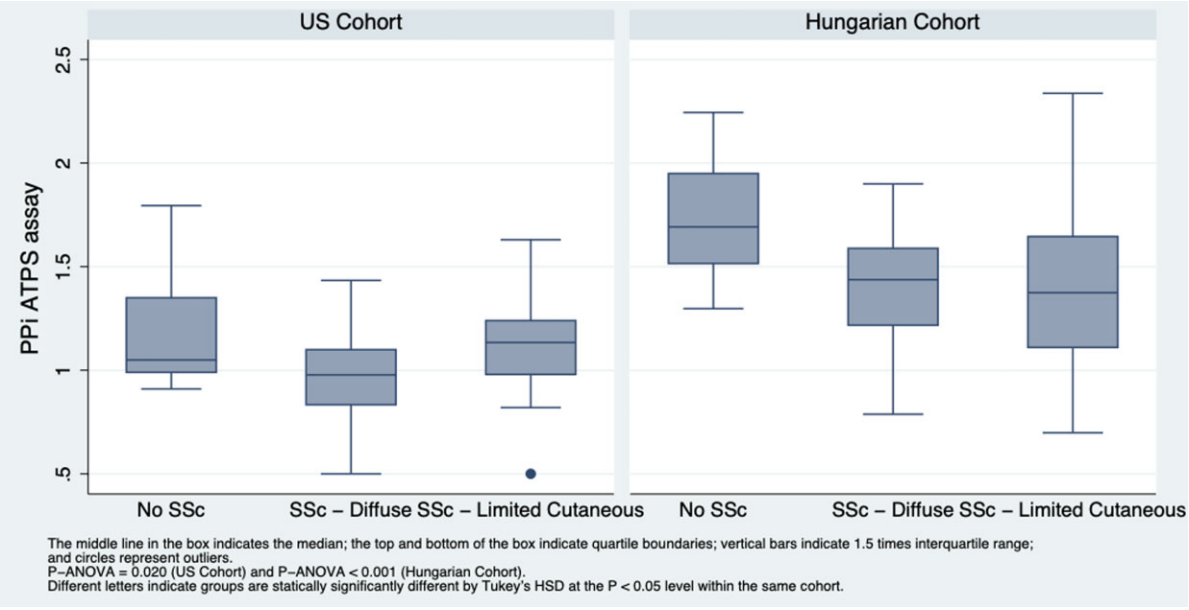
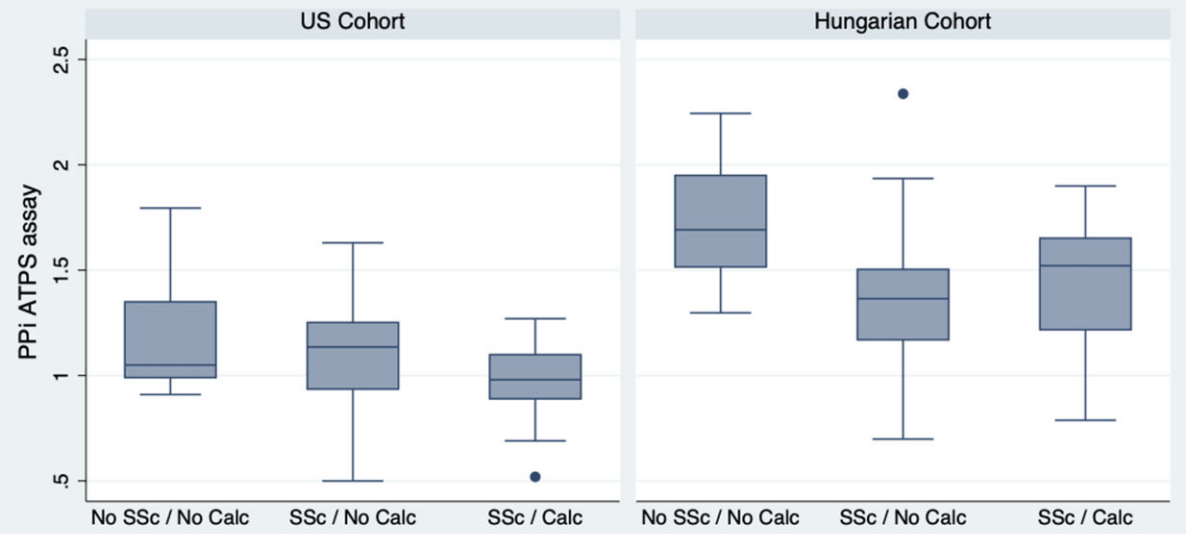


Fig. 2 Distribution of PPi values by calcinosis status for the US (left) and Hungarian (right) cohorts



The middle line in the box indicates the median; the top and bottom of the box indicate quartile boundaries; vertical bars indicate 1.5 times interquartile range; and circles represent outliers.
P–ANOVA = 0.003 (US Cohort) and P–ANOVA < 0.001 (Hungarian Cohort).
Different letters indicate groups are statically significantly different by Tukey's HSD at the P < 0.05 level within the same cohort.

without SSc, and hypothesize that PPi deficiency may contribute to calcinosis formation in SSc. Interestingly, plasma PPi levels were uniformly reduced for both patient groups; i.e. were not statistically different between SSc patients with or without calcinosis (US cohort $P = 0.161$, Hungarian cohort 0.665) (Fig. 2). There were few patients with more proximal (shoulders) or extensive truncal involvement who had similarly very low circulating PPi levels. We rationalize those SSc patients with low circulating PPi levels without calcinosis will eventually develop calcinosis. Conversely, these SSc patients could have had occult calcinosis that were missed. This late complication can also be seen in patients with PXE, whose ectopic calcification is associated with a genetic deficiency (with 100% penetrance) resulting in markedly reduced plasma PPi (50% or less) present from birth, and who typically will not show their ectopic calcification/calcinosis symptoms before the late childhood or teen years [14, 15].

Previous reports confirmed that SSc-calcinosis is most commonly found in the soft tissues or attached to ligaments and tendons [29] and that hydroxyapatite is the primary inorganic component of SSc-calcinosis [30]. Risk factors include longer disease duration, the presence of digital ulcers, acro-osteolysis, having the anticentromere antibody, as well as recurrent trauma or direct pressure over extensor surfaces of the extremities and trunk where many of these deposits are found; additionally, chronic tissue hypoxia and inflammation have been proposed as important mechanisms [30–32]. We propose that the small number of SSc patients may have limited our ability to see differences in PPi levels. Alternatively, while circulating PPi levels may contribute to vascular calcification, they may not reflect what occurs in the soft tissues where calcinosis occurs. Nevertheless, the reduced plasma PPi levels in SSc led to the suggestion that administration of PPi to patients could counteract the ectopic mineralization. Dedinszki *et al.* [28] have shown that orally administrated PPi inhibits soft tissue calcification. As a potent inhibitor of mineralization, PPi may be deficient in other clinical conditions accompanied by ectopic mineralization. The first comparison of circulating PPi levels and vascular calcification was performed in a cohort of patients with advanced kidney disease undergoing haemodialysis, peritoneal dialysis or no dialysis. Specifically, plasma PPi level was negatively associated with vascular calcification in patients with end-stage renal disease and stage 4 chronic kidney disease [27]. Reduced plasma PPi was also found in nephrocalcinosis [33] and aortic valve calcification [34, 35].

A limitation of our study is that the PPi assay has not been standardized, and measurement of circulating PPi levels in the US cohort were consistently lower than those of the Hungarian cohort using the ATPS method. This was likely due to a difference in collection of plasma using tubes with EDTA in the US cohort *vs* tubes without EDTA in the Hungarian cohort. Although this may explain the differences in PPi values between the two cohorts, this does not change the observed significant differences between the SSc and control subjects, irrespective of which cohort was studied. Additionally, not all SSc subjects without calcinosis had imaging of the hands and deeper deposits could have been missed.

The mechanism underlying the reduction of circulating PPi in SSc is currently unknown. Numerous factors

regulate circulating PPi levels. ATP is released from cells by different mechanisms, including ABCC6 transporter [15, 36–38], and PPi is produced from ATP hydrolysis by the enzyme ectonucleotide pyrophosphatase/phosphodiesterase 1 [16, 17]. PPi is also regulated by the ANKH protein, which transports ATP outside of cells to the extracellular matrix where it is enzymatically converted to PPi [39]. On the contrary, the degradation of PPi to phosphate by tissue-nonspecific alkaline phosphatase and other enzymes would then lead to an increased risk of calcification. Thus, PPi homeostasis is regulated by a balance between ABCC6, ENPP1 and ANKH, which promote PPi generation, and the opposing action of tissue-nonspecific alkaline phosphatase catalyzing the degradation of PPi. A study evaluated sequence variants in *ALPL*, *ENPP* and *ANKH* genes encoding PPi metabolizing proteins tissue-nonspecific alkaline phosphatase, ENPP1 and ANKH. The c.1190-65C>A in *ALPL*, c.313 + 9G>T in *ENPP1*, and p.A98A genotype TT in *ANKH* were genetic risk factors in PXE patients [37]. Therefore, it has been suggested that defects in any of the proteins involved in the formation, transport and hydrolysis of PPi can have profound effects on the level of mineralization. Ding *et al.* [40] reported significantly lower nucleoside triphosphate pyro-phosphohydrolase activity, a critical ecto-enzyme hydrolyzes extracellular ATP to produce PPi, in their SSc patients with calcinosis. No genetic studies have been performed yet to suggest any heritable predisposition for calcinosis in SSc.

Environmental influences from a long-standing disease may be important. Host *et al.* [41] reported a significant association between calcinosis severity and prolonged proton pump inhibitor exposure, which is commonly prescribed to virtually all patients with SSc-related oesophageal reflux. Aberrant fibrosis related to the accelerated ageing process has also been proposed in those with chronic diseases such as scleroderma [42], and defective clearance of senescent cells due to disrupted immune regulation and mitochondrial dysfunction have been described in those with pulmonary fibrosis [43]. We propose that similar cellular oxidative stress may occur in the subcutaneous, musculoskeletal tissues of those with advanced, long-standing scleroderma that could accelerate the pathway towards dystrophic calcification [44]. More studies will be needed to delineate other contributing factors, including the reasons for the dysregulated PPi transporter system, other systemic and local changes in the micro-environment that perpetuate this aberrant repair process within the connective tissues, most of which we do not yet understand [44].

Conclusion

Our findings indicate that circulating PPi levels are significantly reduced in SSc patients with or without calcinosis. This may be due to genetic or environmental influences or both, which may be important in the pathophysiology of calcinosis. This aberrancy may contribute to the increasing damage within the soft tissues seen in those with this chronic disease, with calcinosis being one of the manifestations. The reduced circulating plasma PPi levels suggests that administration of PPi to SSc patients could be a therapeutic strategy and larger clinical studies are planned to confirm our findings.

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Data availability statement

The data underlying this article are available in the article and in its online supplementary material.

Supplementary data

Supplementary data are available at *Rheumatology* online.

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