The fibrinolytic system components are increased in systemic sclerosis and modulated by Alprostadil (alpha1 ciclodestryn)

F. Bandinelli[1], F. Bartoli[1], F. Perfetto[1], A. Del Rosso[1], A. Moggi-Pignone[1], S. Guiducci[1], M. Cinelli[1], C. Fatini[2], S. Generini[1], A. Gabrielli[3], R. Giacomelli[4], S. Maddali Bongi[1], R. Abbate[2], M. Del Rosso[5], M. Matucci Cerinic[1]

[1]Department of Medicine, Division of Rheumatology and [2]Dipartimento del Cuore e dei Vasi, University of Florence; [3]Istituto di Clinica Medica Generale, Ematologia ed Immunologia Clinica, Università Politecnica delle Marche, Ancona; [4]Department of Internal Medicine, University of L'Aquila, L'Aquila, Italy; [5]Dipartimento di Patologia e Oncologia Sperimentali, University of Florence, Florence, Italy.

Abstract
Objectives
To evaluate urokinase plasminogen activator (u-PA), urokinase plasminogen activator soluble receptor (su-PAR), plasminogen activator inhibitor I (PAI-1) and tissue plasminogen activator (t-PA) plasma levels in SSc patients (pts) versus healthy controls and their modulation by intravenous alphacyclodestrine (Alprostadil).

Methods
Plasma levels of u-PA, su-PAR, PAI-1 and t-PA were measured in 40 SSc (34 lSSc and 6 dSSc) pts and in 30 healthy controls. In SSc, blood was drawn before and after 3 consecutive daily of Alprostadil infusion (60 mg in 250 cc NaCl 0.9%).

Results
In SSc su-PAR basal levels were higher than controls (7.48 ± 2.5 vs 4.69 ± 0.4 ng/ml; $p = 0.001$) and were significantly reduced by Alprostadil (5.93 ± 1.7; $p = 0.002$), but remain higher than controls ($p = 0.03$). u-PA basal levels were higher than controls (3.78 ± 1.5 vs 1.29 ± 0.3 ng/ml; $p < 0.001$) and were reduced by Alprostadil (2.39 ± 1.7; $p < 0.001$) to control levels. SSc PAI-1 basal levels were lower than controls (31.60 ± 7.7 vs 48.30 ± 6.8 ng/ml; $p < 0.001$) and increased by Alprostadil (34.66 ± 5.4; $p = 0.04$), but lower than controls ($p < 0.001$). SSc t-PA basal levels were higher in respect to controls (1645.81 ± 792.7 vs 571.95 ± 75.5 pg/ml; $p < 0.0001$) and reduced by Alprostadil (1318.06 ± 603.5; $p = 0.04$), but still higher than controls ($p = 0.001$).

Conclusion
Fibrinolysis were increased in SSc. Infusions of Alprostadil modulate u-PA, su-PAR, PAI-1 and t-PA, restoring near normal levels. In SSc, fibrinolysis system may become a potential target for new therapies.

Key words
Fibrinolysis, systemic sclerosis, prostaglandins.

Francesca Bandinelli, MD; Francesca Bartoli, MD; Federico Perfetto, MD; Angela Del Rosso, MD; Alberto Moggi-Pignone, MD; Serena Guiducci, MD; Marina Cinelli, MD; Cinzia Fatini, MD; Sergio Generini, MD; Armando Gabrielli, MD; Roberto Giacomelli, MD; Susanna Maddali Bongi, MD; Rosanna Abbate, MD; Mario Del Rosso, MD; Marco Matucci Cerinic, MD.

Please address correspondence to: Professor M Matucci Cerinic, Department of Medicine, Division of Rheumatology, University of Florence, Viale G. Pieraccini no. 18, 50139 Florence, Italy.
E-mail: cerinic@unifi.it

Introduction

Systemic sclerosis (SSc) is a connective tissue disease of unknown aetiology, characterized by microvascular involvement, immune alteration and fibrosis of the skin and internal organs. In SSc, microvessel abnormalities are characterized by thickening of the intima, thinning of the media, fibrosis of the adventitia and occlusion of vascular lumen (1), due to enhanced thrombus formation and extensive fibrin deposition.

In SSc, the pathway leading to fibrin deposition is not well understood, but several studies suggest that coagulation system is activated, as shown by increases of Thrombin-Antithrombin (TAT) complexes, prothrombin releases fragment 1+2 (F 1+2), and D-dimers circulating levels (2-4). Likewise, controversial issues concern the fibrinolytic activity in SSc. Several studies have reported both depressed basal (5, 6) and activated fibrinolytic activity (7), while others have shown normal fibrinolytic activity (8, 9) and normal tissue plasminogen activator (t-PA) concentrations in skin and plasma (10).

Alprostadil, an analogue of Prostaglandin E_1 (PGE1) with potent vasodilating actions, has been successfully employed by intravenous infusions in several diseases for the treatment of chronic critical limb ischemia (11) and, in SSc, for the treatment of Raynaud phenomenon (RP) (12) and for the control of fingertips and lower limbs ulcers. The increase of peripheral blood flow observed after intravenous Alprostadil may be explained not only by its vasodilating actions, but also by inhibition of platelet adhesion and aggregation and modulation of neutrophil activation (13).

In patients with RP secondary to SSc, intravenous infusions of Alprostadil improve endothelial cells (EC) function as indicated by the decrease of plasma levels of tissue plasminogen activator (t-PA), von Willebrand factor (vWF), and Intercellular Adhesion Molecule 1 (ICAM-1) (14).

On the basis of these considerations, we attempted to evaluate the levels of components of fibrinolytic system in patients with SSc and their acute modulation by an intravenous infusion of Alprostadil.

Therefore, molecule markers of the fibrinolytic system such as tPA, PAI-1, by urokinase plasminogen activator (u-PA) and its soluble receptor, the urokinase plasminogen activator receptor (su-PAR), that regulate extra cellular proteolysis, chemotaxis, cell attachment, proliferation, differentiation and fibrin degradation (15) were determined before and after 3 consecutive days of Alprostadil treatment.

Patients and methods
Patients

Forty consecutive Caucasian SSc patients (38 females and 2 males; mean age: 60.6 ± 9.3 years), attending the section of Rheumatology of the University of Florence, and of the Departments of Medicine of University of Ancona and L'Aquila, were enrolled in the study. Thirty healthy subjects, matched for sex and age with the patients, served as controls. The local ethical committee approved the study and a written informed consent was obtained from both patients and healthy controls. Patients were classified in limited SSc and diffuse SSc according to Le Roy *et al.* (16).

Exclusion criteria were: age < 18 years, pregnancy, stroke in the 4 months preceding the study, myocardial ischemia, heart failure, systemic arterial hypertension not pharmacologically controlled, thrombocytopenia (platelet count < $100,000/10^{12}$ L), thrombocytosis (platelet count > $500,000/10^{12}$ L), renal failure, chronic hepatitis, diabetes mellitus and malignancy.

SSc patients treated with drugs potentially able to modify the evolution of the disease (corticosteroids, methotrexate, cyclophosphamide, D-penicillamine, iloprost) were excluded as well as patients whose conditions did not allow a complete pharmacological wash-out (patients with severe ulcers, severe artery pulmonary hypertension, severe respiratory failure, congestive heart failure III–IV class of NYHA, creatinine values 1.5 mg/dl and megaoesophagus and/or malabsorption).

All SSc patients were under treatment with topical gliceril nitrate, ACE inhi-

bitors, calcium channel blockers, proton pump inhibitors and clebopride. Before sampling, patients were washed out for 10 days from oral and topics vasodilators.

The SSc patients were evaluated for disease duration, calculated from the onset of the first non-Raynaud's symptom, (17) and assessed for microvascular, skin and organ involvement by a careful workout.

Analyses
Skin and microvascular involvement. At the time of blood drawing, the presence of fingertip ulcers, other skin ulcers (e.g. at heels, legs, elbows, forearms), calcinosis, teleangectasias, and Raynaud's phenomenon was recorded. Skin involvement was assessed and scored with the modified Rodnan skin score (18). The small vessel architecture was studied by nailfold videocapillaroscopy (NVC) and patients divided into three groups as follow according to Cutolo *et al*. criteria in early (14 patients), active (14 patients) and late (12 patients) (19).
Cardiovascular involvement. Two dimensional ultrasound evaluation and standard EKG assessed cardiovascular involvement Arterial blood pressure was recorded (1).
Lung involvement. Lung involvement was evaluated by forced vital capacity (FVC), diffusing lung capacity for carbon monoxide (Dlco), and high resolution computed tomography (HRCT)
Kidney involvement. Kidney involvement was evaluated by renal function tests (including 24-h creatinine clearance)
Autoantibodies. Antinuclear antibodies (ANA) (by indirect immunofluorescence on rat liver), anticentromere antibodies (ACA) [by indirect immunofluorescence on Hep-2 cells and by Enzyme-linked immunoabsorbent assay (ELISA) for CENP antigen)] and antitopoisomerase I antibodies (anti-Scl70) (by immunoblot analysis), Rheumatoid Factor (RF) (by ELISA) were determined.

Assessment of fibrinolytic system components
In SSc patients, blood was drawn, be-

fore infusions and after 3 consecutive daily infusions (60 g in 250 cc of physiological solution in 3 hours) of Alprostadil (Alprostadil–Leiclodedrin®, Schwarz Pharm).

After drawing, blood samples were collected in vacutainers containing EDTA (1 mg/ml), maintained in ice for 30-60 minutes, centrifuged (5000 g for 15 minutes) at 4°C to obtain plasma and conserved at -80° C, until assayed.

u-PA (ng/ml), su-PAR (ng/ml), PAI-1 (ng/ml) and t-PA(pg/ml) plasma levels were determined by ELISA kits (kits provided by IMUBIND American Diagnostica, Montreal, Canada). The results were correlated to a standard curve, within the range of linearity. Each sample was evaluated in triplicate and with two different dilutions.

The sensibility levels were: 10 pg of uPA/ml of sample; 0.1 ng of uPAR/ml of sample; 1 ng of PAI-1 /ml of sample. tPA plasma (pg/mL) levels were determined by ELISA kits (kit provided by Bender MedSystems, Vienna, Austria) The sensibility levels were 16 pg/ml.

Statistics
Data were analysed using SPSS 10.0 for Windows. Descriptive statistics were expressed as mean ± standard deviations (SD).

Normal distribution of each examined parameter was verified by Kolmogorov-Smirnoff test. The statistical significance of the differences between means of two groups was evaluated by the Student's t-test for paired or unpaired

Table I. Anthropometric and clinical characteristic of SSc patients and healthy controls.

		SSc (40 patients)	Healthy controls (30 subjects)
Age (years)		60.6 ± 9.3	56.20 ± 11.4
Height (cm)		159.7 ± 7.0	164.2 ± 5.2
Weight (kg)		62.50±13.0	64.8 ± 15.2
BMI		24.1 ± 3.9	26.3 ± 5.4
Sex (Males/Females)		2/40 38/40	1/30 29/30
Subset	lSSc dSSc	34/40 6/40	
R. P.		40/40	
Disease duration (years)		8.8 ± 7.6	
Skin score		12.7 ±10.2	
Skin ulcers	+	6/40	
Fingertip ulcers	+	11/40	
Teleangectasias	+	28/40	
Calcinosis	+	6/40	
Capillaroscopy	Early Active Late	14/40 14/40 12/40	
Autoantibodies	ANA+ Scl-70 + ACA+ auto-Ab - RF +	37/40 10/40 21/40 3/40 2/40	
FVC (%)		99.0 ±23.2	
DLCO (%)		63.6 ±23.0	
Lung HRCT	+	19/38	
Heart involvement	+	15/38	

BMI: body mass index(weight in Kg/ height in m^2; lSSc: limited SSc; dSSc: diffuse SSc; R.P. Raynaud Phenomenon; ANA: antinuclear antibodies; Scl70: anti-Scl70 antibodies; ACA: anticentromere antibodies; auto-Ab-: negativity for autoantibodies; RF: rheumatoid factor; FVC: forced ventilatory capacity; DL$_{CO}$: diffusing lung capacity for carbon monoxide HRCT: high resolution computed tomography.

data and, when indicated, by the Wilcoxon's signed-rank test (paired data) or the U-test of Mann-Whitney (unpaired data).

The statistical significance of the differences between means of more than two groups was evaluated by ANOVA with Bonferroni correction test and Kruskal Wallis test when indicated.

Non-parametric and parametric correlation analyses were performed with the Spearman's rank correlation test and Pearson test, respectively.

A p level of 0.05 or less was considered statistically significant.

Results

Demographic and clinical features of SSc patients (34 lSSc and 6 dSSc) are reported in Table I.

Baseline plasma levels of u-PA, su-PAR, t-PA and PAI-1 (Table II and Fig. 1)

The circulating plasma levels of u-PA, su-PAR, PAI-1 and t-PA in SSc, in d-SSc, lSSc and controls are shown in Table II.

u-PA basal concentrations (3.78 ± 1.5 ng/mL) were significantly higher in SSc than in controls (1.29 ± 0.3; $p < 0.0001$) and both in lSSc (3.41 ± 1.15) and dSSc (5.83 ± 0.68) than in controls ($p < 0.0001$). u-PA levels were significantly higher in dSSc than in lSSc ($p < 0.0001$).

su-PAR basal levels were higher (7.48 ± 2.5 ng/mL) in SSc ($p<0.001$) and in lSSc (7.54 ± 2.48; $p<0.005$) than controls (4.69 ± 0.4), but non in dSSc (7.18

± 2.98). No difference was detected between lSSc and dSSc.

t-PA plasma levels were significantly higher in SSc (1654.8 ± 792.7 pg/mL) and in lSSc (1714.5 ± 809.5) than controls (571.9 ± 75.5) ($p < 0.0001$), but not in dSSc levels (1256.3 ± 602.3). No difference was present between lSSc and dSSc.

PAI-1 levels (31.6 ± 7.7 ng/mL) were significantly lower in SSc patients ($p < 0.0001$) and both in dSSc (27.15 ± 8.29) and in lSSc (32.33 ± 7.48) versus controls (48.3 ± 6.85; $p < 0.0001$). No difference was found between lSSc and dSSc.

Plasma levels of u-PA, su-PAR, t-PA and PAI-1 after Alprostadil (Table II and Fig. 1)

In SSc patients, u-PA plasma levels were reduced after Alprostadil (2.39 ± 1.73 after) in respect to basal values (3.78 ± 1.5; $p < 0.001$), reaching values not different from those of controls (1.29 ± 0.3).

Alprostadil reduced u-PA plasma levels both in lSSc (3.41 ± 1.15 before vs 1.89 ± 0.21 after; $p<0.0001$) and in dSSc (5.83 ± 1.8 before vs 5.05 ± 0.68 after; $p < 0.01$), reaching control levels (1.29 ± 0.3; $p<0.0001$) in lSSc but not in dSSc.

su-PAR plasma levels were reduced by Alprostadil (7.48 ± 2.5 before vs 5.93 ± 1.78 after; $p<0.01$), but remained higher than in controls (4.69 ± 0.4; $p< 0.05$), while, both in lSSc and in dSSc, su-PAR levels (5.51 ± 0.28 and 6.42 ± 2.66, respectively) were reduced in respect to baseline (7.54 ± 2.48; $p< 0.0001$ and

7.18 ± 2.98; $p<0.05$, respectively).

t-PA levels were reduced by Alprostadil (1645.8 ± 792.7 before vs 1318.06 ± 603.55 after; $p<0.05$), but remained significantly higher than controls (571.9 ± 75.5; $p<0.001$). Alprostadil reduced significantly t-PA levels in lSSc (1714.5 ± 809.5 before vs 1360.26 ± 627.72 after; $p< 0.0001$), which however were higher than controls (571.9 ± 75.5; $p<0.01$). In dSSc, t-PA levels did not change.

Alprostadil increased PAI-1 levels (31.6 ± 7.7 before vs 34.66 ± 5.4 after $p < 0.05$), which remained still lower than controls (48.3 ± 6.85; $p < 0.0001$), and did not modify PAI-1 levels neither in lSSc nor in dSSc.

Correlations of u-PA, su-PAR, t-PA and PAI-1 levels with clinical features

u-PA levels were significantly lower in SSc patients with teleangectasias (mean 3.4 ± 0.2 ng/ml) than in patients without (4.6 ± 0.4 ng/ml, $p < 0.02$) and correlated with skin score ($r = 0.3532$; $p < 0.05$).

PAI-1 levels resulted significantly lower in patients without than in those with fingertips ulcers.

No other significant correlation of u-PA, su-PAR, t-PA and PAI-1 levels with age, disease duration, ulcers, lung, heart kidney involvement and autoantibodies pattern was found.
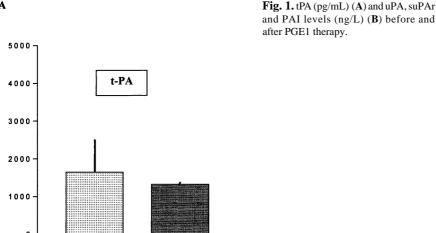
Discussion

The role of fibrinolytic system in the pathogenesis of micro-vessel injury in SSc is still a matter of debate, since many studies have shown discordant

Table II. Circulating levels of fibrinolytic system components in SSc, dSSc, lSSc and controls. Laterally, effects of Alprostadil: levels of significantly in SSc before and after PGE1 therapy.

Pts N°			u-PA(ng/mL)		su-PAR (ng/mL)		t-PA(pg/mL)		PAI-1 (ng/mL)
SSc 40	Before		3.78 ± 1.5 ***		7.48 ± 2.5 **		1645.8 ± 792.7***		31.6 ± 7.7***
	After	**	2.39 ± 1.73	*	5.93 ± 1.78*	*	1318.06 ± 603.55**	•	34.66 ± 5.4***
lSSc 34	Before		3.41 ± 1.15***		7.54 ± 2.48*		1714.5 ± 809.5***		32.33 ± 7.48*** ns
	After	***	1.89 ± 0.21 ns	***	5.51 ± 0.28 ns	***	1360.2 ± 627.72*	ns	33.93 ± 4.96***
dSSc 6	Before		5.83 ± 1.8***		7.18 ± 2.98 ns		1256.3 ± 602.3 ns		27.15 ± 8.29***
	After	*	5.05 ± 0.68***	•	6.42 ± 2.66 ns	ns	1092 ± 424.7 ns	ns	38.58 ± 6.42***
Controls 20			1.29 ± 0.3		4.69 ± 0.4		571.9 ± 75.5		48.3 ± 6.85

***: $p < 0.0001$; **: $p < 0.001$; *: $p < 0.01$; • : $p < 0.05$; Ns: not significative.

A

B

Alprostadil, in SSc patients, significantly reduces the frequency of RP attacks and reduces the levels of endothelial cell damage markers (28-31, 14).

Fibrinolysis is primarily a function of the endothelium and circulating levels of tPA u-PA, su-PAR and PAI-1 are considered a surrogate markers of endothelial derangement. tPA is an endothelial product that increases when endothelium is damaged or activated (32).

During the formation of a blood clot after injury to a blood vessel, fibrinogen is converted into fibrin, where its main function is to strengthen the clot. Excessive fibrin deposition is prevented by the fibrinolytic system through plasmin function, which is responsible for the removal of inappropriately formed fibrin. Plasmin is produced, where required, by cleavage of its inactive precursor, plasminogen, and the rate of this cleavage is driven by the relative proportion of activators and inhibitors of fibrinolysis.

Our data suggest that all components of fibrinolytic system are activated in SSc patients, as indicated by the increased plasma concentrations of u-PA, su-PAR, and t-PA and by the reduced concentrations of PAI-1. In SSc the activation of fibrinolysis may reflect and follow an enhance coagulation cascade suggested by increased levels of vWF, (32,8) fibrinogen, (33), F1-2 fragments, D-dimers and TAT complexes (2-4). Furthermore, at the best of our knowledge, no study supports a primary activation of fibrinolytic system in SSc. From this point of view, the enhanced fibrinolysis observed in our SSc patients may be explained as an attempt to limit the engulfment of the micro vessels with fibrin and, eventually, their occlusion. Interestingly, chronic thrombin generation and intra-vessels fibrin deposition may have consequences beyond their haemostatic role. Thrombin may enhanced fibroblast replication and chemotaxis (34,35) and may favour the synthesis and release endothelin-1, a vasoconstrictive and profibrotic peptide, that play a pivotal role in the pathogenesis of SSc. In close relationship with thrombin, fibrin

results. An activation of the fibrinolytic system in SSc, characterized by increase of tPA (14, 20) and D-dimers (21, 22) or by reduction of inhibitors such as 2-antiplasmin, has been reported (23). Instead, a reduction of some fibrinolytic components such as tPA and uPA (23, 24) or an increase of PAI-1 levels (2, 25, 26) has been shown by other authors. Other studies reported both activation and depression of the fibrinolytic system with increase of t-PA and PAI-1 concentrations (27) or increased levels of some fibrinolytic components associated to a reduction of fibrin degradation products (D-dimers) (4). Herrick *et al.* (8) found no significant variation of PAI-1 and t-PA levels in patients with SSc. All these discrepant findings show the difficulty in defining the changes of the fibrinolytic system in SSc.

In our patients, before Alprostadil treatment, the fibrinolytic system seems activated and not down regulated. However, Alprostadil infusion induces a significant decrease of t-PA, u-PA and su-PAR levels and a significant increase of PAI-1 concentrations, indicating a blunting of the fibrinolytic activity.

Since SSc is characterized by widespread fibrin deposit, the down-regulation of fibrinolysis just after the Alprostadil infusion might be considered an unfavourable drug effect. Nevertheless, a bulk of evidence indicates that

may induce vWF release from endothelial cells (36) and may promote fibroblast proliferation and chemotaxis (37). In our SSc patients, Alprostadil decrease t-PA and su-PAR levels, restore normal u-PA levels and increase PAI-1 levels. This suggests a positive action of the drug on the endothelial injury reducing the imbalance of the homeostasis between the procoagulant and fibrinolytic properties of endothelial cells (1).

The protective action of Alprostadil on the endothelium may acts through different pathways, like the protection against the reperfusion injury, the damage of reactive oxygen species, the protease neutrophil cytotoxicity, and by the inhibition of leukocyte adherence to endothelium (38). Alprostadil modulates haemostatic and fibrinolytic parameters. In patients with intermittent claudicatio, it reduces thrombin formation and fibrin degradation, decreasing plasma levels of all haemostatic and fibrinolytic parameters (13). Moreover, Alprostadil significant decreased the tPA plasma levels in SSc patients (14) at the same doses used in our patients but with different time schedule (infusion was repeated on 5 consecutive days, 3 times at 6 week intervals during the winter months). Our data shows also an increased concentration of su-PAR, usually found in patients with various forms of malignancies (39-43) or severe form of infections (44-47). suPAR is derived from proteolytic cleavage of uPAR from the cell surfaces by a number of proteases, such as chymotrypsin (48), phospholipase C (49) and uPA (50). uPAR is expressed on different cell types, including neutrophils, lymphocytes, macrophages, endothelial and malignant cells and with its ligand, uPA, is involved in numerous biological functions. In the pathogenesis of cancer, uPAand uPAR play a key role in tissue invasion by converting plasminogen into plasmin, leading to the degradation of extracellular matrix (51). Moreover uPAR is able to bind -integrins (52), promoting the migration of leukocytes (53). Proteolytic cleavage of uPAR from the cell surface can release an active chemotactic form of suPAR (54,55). Inter-

leukin-1 , basic fibroblast growth factor or vascular endothelial growth factor increase suPAR release from endothelial cells, whereas platelet derived growth factor-BB, bFGF or IL-1 stimulate suPAR release from vascular smooth muscle cells (56). Immune electron microscopy indicates that, in atherosclerotic vessels, suPAR may be found on cell membranes as well as in the extracellular matrix. These findings may indicate that, in SSc patients, suPAR from vascular cells is upregulated by fibrinolityc activity, by proangiogenic as well as pro-atherogenic growth factors and cytokines, and may be deeply involved in microvascular abnormalities.

Although an analysis of the fibrinolytic variables in different subsets of disease was not the main purpose of this study, we found that, after Alprostadil treatment, uPA and, to a lesser extent, suPAR plasma levels remained still higher in patients with dSSc than in patients with lSSc (Table II). Whether the increased plasma levels of uPA and suPAR may be specific markers of dSSc or, simply, may reflect the extension of the disease in a wide skin area and/or to internal organs in this subset is, at present, unknown. The positive relationship between uPA plasma levels and the skin score seems to confirm these data. However, our results suggest that the capability of Alprostadil to restore the endothelium damage and, consequently, to reduce the fibrinolytic activity is more effective in lSSc than in dSSc.

In conclusion, these data indicate that fibrinolysis is activated in SSc and that the positive clinical effects of Alprostadil, affecting several different mechanisms, may also lead to a reduction of fibrinolityc activity. Our results further support the frequent use of Alprostadil in the management of SSc before endothelial damage became irreversible.

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