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Pupillocynetic Activity of Substance P in Systemic Sclerosis

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ABSTRACT. ***Objective.*** In systemic sclerosis (SSc), dysfunctions of peripheral nervous system (PNS) have been observed. Substance P (SP) instillation in human eye induces a cholinergic-independent pupil myosis. Pupil basal diameters (PBD) and pupil responsiveness to SP, expressed as area under the curve (AUC), were studied by pupillometry to assess SP-ergic fiber state and function in SSc.

Methods. Forty SSc patients [24 with limited (lSSc), 16 with diffuse (dSSc) disease] and 40 controls underwent pupillometric evaluation. After evaluation of PBD, SP 10^{-3} M was instilled in one eye and placebo in the contralateral eye. Antinuclear (ANA), anticentromere (ACA), and anti-Scl-70 autoantibodies were correlated with PBD and AUC.

Results. PBD was significantly lower in SSc patients versus controls ($p < 0.001$). PBD was minor in lSSc versus both dSSc and controls ($p < 0.05$), but no difference was found between dSSc and controls. In SSc, SP 10^{-3} M induced greater myosis compared to controls ($p < 0.001$). SP 10^{-3} M-induced myosis was higher in lSSc versus both dSSc and controls ($p < 0.05$). ACA significantly correlated with decreased values of PBD and AUC ($p < 0.001$).

Conclusion. Our results show that PBD is reduced in patients with SSc and that SP induces a more intense myosis in SSc than controls. Moreover, in lSSc PBD is lower and SP increases the myosis in lSSc compared to dSSc and controls. This suggests a peculiar dysfunction of PNS in patients with the limited subset of SSc. (J Rheumatol 2003;30:1231–7)

Key Indexing Terms:
SYSTEMIC SCLEROSIS PUPILLOMETRY SUBSTANCE P
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Systemic sclerosis (SSc) is characterized by microvascular involvement and abnormal immune and fibroblast activation, which results in fibrosis of skin and internal organs [heart, lungs, gastrointestinal (GI) tract, kidney][1]. In SSc, peripheral nervous system (PNS) involvement is frequent, ranging from 16 to 35% of cases[2], and impairing both the function of cranial (mainly trigeminal) and spinal nerves (motor and sensory polyneuropathies and median nerve neuropathy)[2,3]. Involvement of the autonomic nervous system (ANS) seems to be characterized by the increase of sympathetic nervous system activity and reduction of parasympathetic function[4]. ANS impairment is involved in the genesis of Raynaud's phenomenon[2,5,6], in R-R tract modifications[7-9], and in GI motility dysfunction[10-12]. In SSc, ANS involvement has been investigated by different methods, such as sympathetic skin response[13], R-R tract evaluation[7-9,14], and esophageal manometry[14].

Recently, ANS function in SSc was also assessed by pupillometry[14,15]. This technique allows study of systems regulating the pupillary cycle (mainly but not exclusively tuned by ANS) and its modulation by stimuli of different origins (physical, chemical, and biological). In SSc, pupillometry has identified a pupillary autonomic neuropathy[14,15] that was correlated with esophageal dysfunction[14].

Substance P (SP) is present in small myelinated Aδ fibers and in small unmyelinated sensory C fibers[16]. Upon different stimuli, SP can be released by sensory terminals and exerts a direct and an indirect vasoactive (inducing vasodilatation and vasopermeabilization), proinflammatory, mitogenic, nociceptive and immune-modulating activity,

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taking part in neurogenic inflammation[17,18]. SP fibers innervate skin, mucosal membranes, GI system, lungs, and joints[19], and SP is a potent mediator of neurogenic inflammation in SSc[5,6].

SP fibers are also present in the iris of mammals[20,21], where they induce myosis[22] acting on the iris sphincter muscle[23].

In the eye, pupil diameter is largely controlled by ANS activity: the sympathetic branch induces pupil dilatation (mydriasis), while the parasympathetic branch causes reduction of pupil diameters (myosis). Similarly, pharmacological or physical stimuli acting on the sympathetic system increase pupil diameter, while stimuli eliciting the parasympathetic branch reduce pupil diameter. The sensory innervation of the iris is provided by the ophthalmic branch of the trigeminal nerve, some of whose fibers are SP-ergic (Figure 1). These fibers have omathropine-resistant[23], non-cholinergic myotic activity[23,24], mediated by 2 different tachykinin (NK) receptors, NK1 and NK3[25-28]. SP induces a dose-dependent myosis in the human eye[29], but does not reduce omathropine-induced mydriasis[23].

The pupil is an accessible site to study the state and function of the different systems controlling pupil diameter, and it may be investigated by various systemic and local pharmacological stimuli. Moreover, pupillometry is a noninvasive, safe, and reproducible technique[23] already proposed for assessment of pupil diameters in SSc without using pharmacological stimulus[14,15].

We assessed pupil function in SSc to evaluate its basal conditions in the different subsets of the disease, and to verify the pupil response of sensory fibers to SP, thus assessing the *in vivo* function of the PNS (SP-ergic fibers) in the pupil.

MATERIALS AND METHODS

Patients and controls. One hundred consecutive patients with SSc out of 202 referred to the Section of Rheumatology of the University of Florence were recruited for the study. Forty (38 female and 2 male) were selected and classified according to Le Roy, *et al*[30] as having the limited subset (lSSc; 24 patients) or the diffuse subset (dSSc; 16 patients) of the disease. They were also evaluated for disease duration (considered from the onset of the first non-Raynaud symptom). Patients' characteristics are shown in Table 1.

Forty controls matched for sex and age with SSc patients (38 female, 2 male; mean age 55.9 ± 8.6 yrs) were also studied. All subjects underwent careful anamnesis and clinical and hematological examinations. Exclusion criteria for both SSc patients and controls included: acute or chronic concomitant illnesses (diabetes mellitus, lung emphysema, blood hyperten-

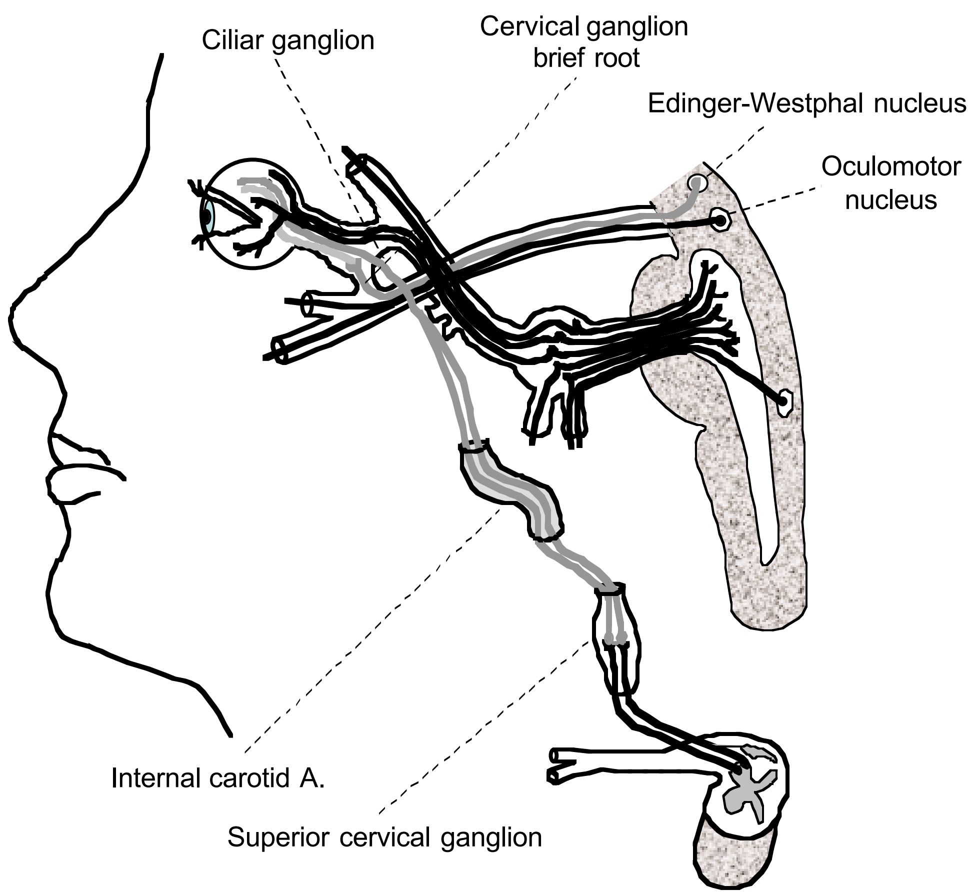


Figure 1. Nerves of the iris: parasympathetic nerves (light grey), sympathetic nerves (intermediate grey), and sensory nerves (black).

Table 1. Demographic and clinical data of SSc patients.

	SSc	lSSc	dSSc
Total, n	40	24	16
Female, n	38	23	15
Male, n	2	1	1
Mean age, yrs, ± SD	57.1 ± 9.3*	58.5 ± 8.2*	54.8 ± 9.1*
Mean duration SSc, yrs, ± SD	10.4 ± 5.7	11.2 ± 6.2**	9.0 ± 2.3**
Autoantibodies			
ANA	38/40	23/24	15/16
Scl70	12/40	1/24	11/16
ACA	19/40	18/24	1/16

* Mean age not different between SSc versus controls, and between lSSc versus dSSc versus controls ($p > 0.05$).
** Mean disease duration not different between patients with lSSc and dSSc ($p > 0.05$). lSSc: limited SSc; dSSc: diffuse SSc; ANA: antinuclear antibodies; Scl-70: anti-topoisomerase I antibodies; ACA: anticentromere antibodies.

sion, etc.) smoking, peripheral neuropathy, treatment by drugs interfering with peripheral and central nervous system (α/ß-agonist/antagonist, parasympathetic stimulatory or inhibitory drugs, antidepressants, antipsychotics, psychotropic substances), and, for patients with SSc, Sjögren's syndrome, inability to undergo a 15-day washout for the presence of severe cutaneous ulcers, pulmonary hypertension, pulmonary alveolitis, renal failure, and severe cardiac or GI involvement. All subjects that, on ophthalmological examination, presented ocular hypertension, cataract, crystalline lens substitution, keratitis, or pupil alterations (such as Claude-Bernard-Horner syndrome, Argyll Robertson pupil, Holmes-Adie pupil, Marcus-Gunn phenomenon) potentially interfering with pupillometric results were also excluded. Before pupillometric assessment, patients underwent a 15-day pharmacological washout and only proton pump inhibitors (15 patients), H_2-blockers (14), and sucralfate (10) were allowed. Systemic nonsteroidal antiinflammatory drugs were also withdrawn.

For controls, any kind of drug was strictly forbidden for 15 days before pupillometric measurement. Both patients and controls were forbidden, for at least 8 hours before the examination, to use alcohol and activators of sympathetic functions, such as coffee, tea, or chocolate. Declaration of Helsinki statements were respected. All subjects were fully informed about the purpose of the study and gave written consent to participate.

Autoantibodies. Antinuclear antibodies (ANA) were determined by indirect immunofluorescence (IIF) on rat liver and HEp-2 cells. Anti-topoisomerase I antibodies (Scl-70) were assessed by double immunodiffusion[31], and by direct and competitive inhibition ELISA[32]. Anticentromere autoantibodies (ACA) were evaluated by IIF, using HEp-2 cell line as substrate.

SP preparation and instillation. Synthetic SP (Sigma, Milan, Italy) was diluted in physiologic solution 0.9% to obtain the concentrations of 135 µg/100 µl, equivalent to 10^{-3} M (SP 10^{-3} M)[23]. During the pupillometric session, all subjects received an ocular instillation of 100 µl of the SP 10^{-3} M solution in a randomly chosen eye, and an equivalent volume of physiologic solution in the contralateral eye.

Pupillometry. Each pupillometric session started at 9:00 am and lasted 120 minutes. The study of pupil diameters was carried out by an electronic computerized pupillometer with dedicated software. Pupillometry was performed in both eyes, the first randomly chosen, with a TV monocular electronic pupillometer (Pupilscan, ASL, Bedford, MA, USA) consisting of an infrared-sensitive television camera, a chin and forehead rest table, a control unit, and a monitor. To assess pupil basal diameter (mm), pupillometry was performed in a soundproof room, after a darkness adaptation of 20 min (light intensity < 1 lx). Before and during measurement sessions, each subject was requested to focus on a standard green light produced by an eye-fix lamp included in the TV camera. A green light served as a standard reference point for accommodation. The distance between the observed eye and the lens was 11 cm and it corresponded to camera focus. The pupil diameter after adaptation to darkness was considered for basal evaluation (time 0). After the basal evaluation of pupil diameter, SP 10^{-3} M was instilled.

After instillation of SP 10^{-3} M and a latency time of 15 min to allow SP 10^{-3} M to exert its pharmacological action[23], 4 measurements of the pupil diameter response (mm) were carried out at intervals of 15 min during the first hour, and 2 measurements at intervals of 30 min during the second hour (times 0', 15', 30', 45', 60', 90', 120'). In the intervals between measurements, the subjects remained in the same standard conditions.

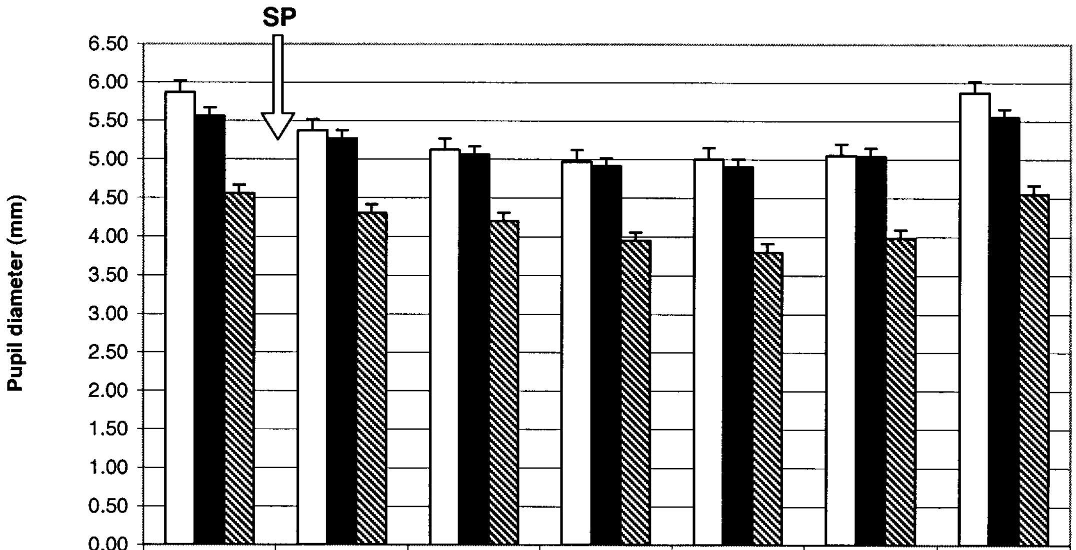
Statistical analysis. Statistical analysis was performed using SPSS for Windows (v8.0, 1997; SPSS Inc., Chicago, IL, USA). Two statistical variables were chosen to evaluate basal pupil diameters and their modifications after SP instillation in controls and patients with SSc, lSSc and dSSc. (1) Pupil basal diameter (PBD): the mean of pupil diameters of all subjects in each group before SP instillation, used to assess the status of iris innervation before SP instillation. (2) Area under the curve (AUC): a summary variable given from the patient-specific series of pupil diameter versus time data points, used to assess the pharmacological action of SP on pupil diameters.

Mean ages and disease durations were expressed as mean ± standard deviation (SD). Pupil diameter values are expressed as mean ± standard error of the mean (SEM). The 2-tailed t test for paired samples was used to compare PBD and AUC of SSc patients (as a whole group) and controls. One-way analysis of variance (ANOVA), assessed with the post-hoc Scheffe test for multiple comparisons, was used to analyze differences among the groups in PBD and AUC. A value of $p < 0.05$ was considered significant. To test if there was a significant association between autoantibody pattern and pupil variables (PBD and AUC), a multivariate regression analysis was conducted. A backward stepwise method of variable elimination was used where variables with $p < 0.10$ were eventually retained in order to explore not only the associations with clear statistical significance ($p < 0.05$), but also those suggested in terms of statistical trends ($0.05 < p < 0.10$). As basal diameters of right and left eyes were not significantly different, only a PBD set was arbitrarily chosen for the comparisons with autoantibodies (see below).

RESULTS

No difference in mean age was found between SSc patients and controls or between lSSc or dSSc and controls. Mean disease duration was not different between lSSc and dSSc (Table 1). Topical application of SP 10^{-3} M caused neither conjunctive reddening nor signs of ocular inflammation.

In Figure 2, data for basal and pupil diameters after SP 10^{-3} instillation in patients with lSSc and dSSc and controls are shown.



	Basal	15'	30'	45'	60'	90'	120'
☐ Controls	5.87	5.37	5.12	4.97	5.00	5.05	5.87
■ dSSc	5.56	5.27	5.06	4.91	4.90	5.04	5.55
▧ lSSc	4.55	4.30	4.20	3.95	3.80	3.98	4.55

Time of single measurements (min)

Figure 2. Pupil diameters before and after instillation of substance P (SP) 10^{-3} M. dSSc: diffuse SSc; lSSc: limited SSc.

Basal pupil diameters. No significant difference in PBD was seen between right and left eyes in controls, the overall SSc group, or patients with lSSc or dSSc.

PBD were significantly smaller in SSc patients compared to controls ($p < 0.001$) (Table 2).

A significant overall difference in PBD between lSSc versus dSSc versus controls was shown by ANOVA ($p < 0.05$). By Scheffe test, PBD of those with lSSc were smaller than controls ($p < 0.001$) and dSSc ($p = 0.001$), while no difference in PBD of those with dSSc versus controls was found (Table 2).

Effect of instillation of SP. SP 10^{-3} M induced a significant major myosis in SSc patients versus controls ($p < 0.001$) (Table 2).

A significant overall difference between those with lSSc versus dSSc versus controls in the response of treated eyes to SP 10^{-3} M instillation (AUC) was found by ANOVA ($p < 0.001$). By Scheffe test, SP 10^{-3} M induced a stronger myosis in lSSc than in dSSc ($p = 0.001$) and controls ($p < 0.001$). In those with dSSc, the myosis induced by SP 10^{-3} M was not significantly different from controls (Table 2).

Correlations between autoantibodies and pupil variables. In patients with lSSc only, a significant correlation between ACA and PBD ($p < 0.001$; partial correlation 0.659) and between ACA and AUC ($p < 0.001$; partial correlation 0.636) was shown by multivariate regression analysis.

In those with lSSc, pupil variables were compared between ACA-positive and ACA-negative patients. PBD of

Table 2. Pupillary variables in SSc vs controls and in dSSc vs lSSc vs controls.

Variables	SSc, mean ± SD	lSSc, mean ± SD	dSSc, mean ± SD	Controls, mean ± SD
PBD	4.91 ± 1.0	4.55 ± 1.0	5.56 ± 0.9	5.87 ± 0.6
AUC	562.7 ± 124.1	503.2 ± 112.2	634.6 ± 59.4	654.0 ± 86.4

	t Test	One-way ANOVA	Post Hoc Paired Contrasts (Scheffe test)		
	SSc vs Controls	Overall Group Effect (lSSc vs dSSc vs Controls)	lSSc vs Controls	dSSc vs Controls	lSSc vs dSSc
	p	p	p	p	p
PBD	< 0.001	< 0.05	< 0.001	0.192	0.001
AUC	< 0.001	< 0.001	< 0.001	0.684	0.001

lSSc: limited SSc; dSSc: diffuse SSc; PBD: pupil basal diameter; AUC: area under the curve due to SP 10^{-3} M instillation.

ACA-negative lSSc patients were not different from PBD of ACA-positive lSSc patients (4.40 ± 0.78 vs 4.61 ± 1.21 mm; $p > 0.05$). Accordingly, AUC in ACA-negative lSSc patients was not different from the AUC of ACA-positive lSSc patients (517.49 ± 108.26 vs 506.75 ± 136.85 mm; $p > 0.05$).

ACA did not correlate with PBD and AUC in those with dSSc, and ANA and anti-Scl-70 did not correlate with any pupil variables in those with lSSc or dSSc (data not shown).

DISCUSSION

Our data clearly show that pupil diameters in SSc are basally reduced, suggesting a functional impairment of the pupil. This dysfunction is corroborated by the evidence that SP stimulation in SSc induces a major myosis.

Indeed, this is the first study demonstrating an altered response *in vivo* to SP-ergic nerve stimulation in SSc.

We can hypothesize that our findings could be due either to hyperactivation of parasympathetic cholinergic and/or sensory SP-ergic fibers, or to a reduced function of the adrenergic sympathetic system.

The interesting finding is that the significant difference found between SSc patients and controls in both PBD and after SP stimulation is mainly due to the modifications detected in lSSc.

Pupil diameter is regulated by a sophisticated push-and-pull mechanism generated by the counteracting action of the sympathetic and parasympathetic branches of the ANS. Therefore, myosis can be the result of different mechanisms: an increased parasympathetic drive as well as a decrease in the sympathetic outflow. On this unbalanced push-and-pull mechanism, a pivotal role can also be played by SP sensory fibers, whose activation can trigger an increased parasympathetic response, as in the case of a denervation supersensitivity. It has been shown that a chronic denervation at the ocular level after the application of an irritating stimulus causes an enhanced sensory activity, due to a hypertrophic sensitive system with increased SP release[33]. SP also inhibits norepinephrine release, in a dose-dependent manner, during electrical nerve stimulation. Thus, SP may also have a modulatory effect on noradrenergic activity and cause a decrease in norepinephrine release from adrenergic neurons[34].

The reduction of pupillary basal diameter in lSSc suggests that a pupil dysfunction is present, and that it may be sustained, at least in part, by an impairment of SP-ergic tone. Accordingly, in lSSc, SP may stimulate a more intense myosis, probably related to the dysfunction of SP-ergic transmission. This could be due either to a hyperactivated status of the SP-ergic system or to a higher parasympathetic activity causing myosis and overwhelming the sympathetic tone that, on its part, can be reduced. Alternatively, as in the eye, a close functional interaction of sensory and autonomic innervation (both parasympathetic and sympathetic branches) exists; both hypotheses could be considered.

In patients with dSSc, the basal iris diameter is not different from controls, although a tendency to decreased basal diameters and a more myotic response to SP instillation may be found. This could indicate that a minimal efficacy of the push-and-pull mechanism is still present in patients with this disease subset, resulting in a range of pupil diameters more similar to controls than to those with lSSc.

It is interesting that Hermosillo, *et al* found a decreased parasympathetic control of heart rate in patients with lSSc, significantly different from those with dSSc[7]. This result seems to support our data, showing a basal difference in pupil diameter between dSSc and lSSc, and to ratify the remarkable difference in PNS involvement between the 2 disease subsets.

In SSc, the pupillary ANS activity has been evaluated[14,15]. Straub, *et al* described a high prevalence of pupillary autonomic neuropathy in 19 SSc patients[15]. To evaluate the sympathetic and the parasympathetic branches of the ANS, the maximal pupil area in darkness and the latency time of the pupil reflex after a light stimulus, respectively, were assessed; 10.5% of SSc patients had pathological pupil latency time and 13.7% presented an abnormal maximal pupil area. These data suggested an impairment in both sympathetic and parasympathetic branches of pupil ANS. In another study, the same group evaluated the association between autonomic dysfunction (at both cardiovascular and pupillary level) and esophageal impairment. Out of 36 SSc patients, esophageal dysmotility was found in 27 and autonomic dysfunction in 12. The autonomic dysfunction was found at the pupillary (6 patients) or the cardiovascular level (5 patients) or both (one patient)[14]. In these reports, the lack of details about SSc subsets does not allow identification of a difference in autonomic balance, at the pupillary level, between lSSc and dSSc. Our study aimed to evaluate the iris response to SP, not to specifically assess pupillary sympathetic and parasympathetic function, although as SP acts, even in a partial way, through a cholinergic pathway[27], an impairment of the parasympathetic branch of the ANS could be indirectly hypothesized. As well, a dysfunction in the ANS sympathetic branch could be speculated, as SP inhibits norepinephrine release from noradrenergic neurons[34].

In our patients, the reduction of basal iris diameter and the myosis induced by instillation of SP 10^{-3} M significantly correlates with ACA in lSSc. By contrast, ANA and anti-Scl-70 did not correlate with pupillary measures.

As far as we know, ACA have never been correlated to PNS and/or to ANS impairment, as suggested for anti-uridine-rich RNA molecule type 1 (U1RNP) and anti-Scl-70[35,36]. In a cohort of 31 patients with SSc, 11 showed PNS involvement (trigeminal neuropathy or polyneuropathy) and 8 of them (73%) had either anti-U1RNP or anti-Scl-70 antibodies. The authors concluded that some subgroups of SSc patients, identified by the presence of U1RNP and anti-Scl-70, could be more prone to develop neurological manifestations[35]. In a recent study on the ANS, esophageal motor dysfunction correlated with anti-Scl-70 in SSc, and also with U1RNP antibodies in patients with mixed connective tissue disease and SSc. Esophageal motor dysfunction was also prevalent in patients with cardiovascular autonomic neuropathy[36].

In our patients, ACA were mainly present in those with lSSc (18/24 patients), and in this subset they correlated both with a reduced pupillary basal diameter and with a more prominent SP-induced myosis. We also found that those with lSSc had a smaller basal diameter and reacted with a stronger myosis to SP instillation compared to those with dSSc. Thus, in lSSc, the presence of ACA seems to correlate with more severe impairment of the pupillary nervous system.

Our data show that patients with lSSc have a reduced pupillary basal diameter and that they respond with a more prominent myosis to the instillation of SP compared to those with dSSc and to controls, and that ACA are correlated with a reduced iris basal diameter and with a more prominent myosis in response to SP instillation. Our data could account for a stronger SP-ergic and/or parasympathetic tone and for a different condition, activation, and response of iris SP fibers in lSSc compared to dSSc, and could confirm that pupillometry is useful to evaluate involvement of the nervous system in SSc. However, further studies on larger groups of patients are needed to determine if pupillometry and ocular instillation of SP may be helpful in early differentiation of lSSc and dSSc.

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