Alteration of microcirculation is a hallmark of very early systemic sclerosis patients: a laser speckle contrast analysis

A. Della Rossa[1], M. Cazzato[1], A. d'Ascanio[1], A. Tavoni[2], W. Bencivelli[2], P. Pepe[1], M. Mosca[1], C. Baldini[1], M. Rossi[3], S. Bombardieri[1]

[1]*Department of Musculoskeletal and Skin Diseases, Rheumatology Unit;* [2]*Immunoallergology Unit and* [3]*Department of Internal Medicine, University of Pisa, Italy.*

Alessandra Della Rossa, MD
Massimiliano Cazzato, MD
Anna d'Ascanio, MD
Antonio Tavoni, MD
Walter Bencivelli, Statistician
Pasquale Pepe, Statistician
Marta Mosca, MD
Marco Rossi, MD
Chiara Baldini, MD
Stefano Bombardieri, MD

Please address correspondence to:
Alessandra Della Rossa, MD,
Department of Muscoloskeletal and Skin Diseases,
Rheumatology Unit,
S. Chiara Hospital,
Via Roma 67,
56126 Pisa, Italy.
E-mail: a.dellarossa@ao-pisa.toscana.it

Received on September 5, 2012; accepted in revised form on October 9, 2012.

Clin Exp Rheumatol 2013; 31 (Suppl. 76): S109-S114.

© Copyright CLINICAL AND EXPERIMENTAL RHEUMATOLOGY 2013.

Key words: Raynaud's phenomenon, systemic sclerosis, laser speckle contrast imager, differential diagnosis

Competing interests: none declared.

ABSTRACT

Objectives. *To investigate blood flow and microvascular reactivity by laser speckle perfusion imager (Perimed, Jarfalla) in consecutive patients affected by Raynaud's phenomenon at baseline and following dynamic stimulations.*

Methods. *Skin blood flow in the dorsum of the hand was measured at baseline and after cold test and post-occlusive hyperaemia test in 56 consecutive subjects affected by Raynaud's phenomenon (RP), 20 primary (PRP) and 36 secondary to systemic sclerosis (SSc). Twenty healthy subjects (HS) were studied as controls.*

Results. *After cold test, SSc had a significant reduction in blood flow (-58%) as compared to HS (-19%) (p=0.01). Recovery time was significantly higher in SSc (58 minutes) as compared to HS (18 minutes) and PRP (19 minutes) (p=0.006 and 0.0016, respectively). Peak flow after ischaemic test was significantly reduced in SSc (+237%) as compared to PRP (+485%) (p=0.0068). Post-ischaemic hyperaemic area under the curve (AUC) was blunted in SSc (79U/sec) compared to PRP (167 U/sec) (p=0.0126). Proximal distal gradient was noticed in 74% of HS, 45% of PRP and 36% of SSc (p=0.01).*

Homogeneous pattern of flux distribution was significantly different between HS (95%), PRP (80%), and SSc (16%) (p<0.0001). Among SSc patients, a significant difference in ischaemic challenge was shown between patients with early-SSc versus patients with definite-SSc.

Conclusions. *Our preliminary results indicate a clear-cut alteration of the dynamic of microcirculation in SSc-RP as compared to PRP and HS. Among SSc patients, early-SSc is a separate entity as compared to established disease.*

Introduction

Raynaud's phenomenon (RP) is a reversible vasospasm of the extremities that can occur either as an isolated symptom without underlying disorders (primary RP) or in association with another disorder or condition (secondary RP). Most cases of severe RP are associated with Connective tissue diseases (CTD). This symptom occurs often in CTD (1-5) and in 90% of patients with systemic sclerosis and is often perceived as the most important clinical problem (6). RP is the clinical reflection of diffuse microvascular damage typical of the disease. Microvascular changes are typically observed in the nailfold by capillary microscopy and are in fact exploited for the early diagnosis of SSc and prognostication of disease evolution (7-9). In fact, according to the so-called vascular hypothesis raised by LeRoy, microvascular involvement is pivotal for SSc pathogenesis and may be the direct trigger for the fibrotic process seen in this disease (10).

Capillaroscopy generally provides static information on microvascular involvement and few data on the dynamic of microvessels are given by this technique. On the other hand, laser speckle perfusion imager (LASCA) may offer the advantage of evaluating microvessel reactivity. This technique has proven reliable and reproducible in measuring skin microvascular reactivity in healthy subjects (11). The aim of the present work was to perform LASCA at baseline and after dynamic stimuli in consecutive patients affected by Raynaud's phenomenon, to outline differences in different Raynaud's populations and to test the performance of LASCA in discriminating primary from secondary Raynaud's phenomenon, with particular reference to SSc-related Raynaud's phenomenon.

Table I. Clinical data of SSc patients.

Patient number	36	
F/M	34/2	
Mean age	55	(18-82)
Disease duration (RP, non RP)	21,7-15,4	
Mean Rodnan's skin score	7	
Digital ulcer history	14/36	(38%)
D/L/early-SSc	4/24/8	
Oesophageal involvement	25/36	(69,4%)
ILD	15/36	(41%)
PHT	3/36	(8,3%)
ACA	23/36	(62,8%)
Scl-70	8/36	(22,8%)

Table II. Epidemiological and clinical features of the subjects.

	SSc-RP	PRP	HS
Sex (F/M)	36/2	16/4	18/5
Mean age	55 ± 15 (18-82)	36 ± 13 (19-64)	42 ± 12 (26-59)
Mean duration of RP (years)	15.7 ± 16	11.5 ± 15	-
Vasodilator therapy	16/36	-	-
Arterial hypertension	6/36	-	-
Diabetes	-	-	-
Hyperlypidemia	3/36	-	-
Smoking	4/36	-	-
BMI (mean)	23.7	21.7	21.3

Table III. Capillaroscopy patterns and capillary density in Raynaud's patients.

	SSc-RP	PRP
Pattern:		
Normal or minimal changes	-	20/20
Scleroderma pattern:	36/36	-
early	11/36	-
active	9/36	-
late	16/36	-
Capillary density (per mm)	4 ± 1.6	7.6 ± 0.5

Patients and methods

The study protocol was approved by the local ethics committee. After written informed consent, a total of 76 subjects were recruited: 20 healthy subjects (HS) and 56 consecutive patients with RP attending the rheumatology clinic in the period from June 2011 to May 2012.

Patients with Raynaud's phenomenon were divided into two groups: 20 with PRP according to LeRoy Criteria (12) and 36 with Raynaud's phenomenon secondary to SSc (SSc-RP). Data of patients with systemic sclerosis are summarised in Table I. Twenty-eight patients fulfilled the American College of Rheumatology criteria for diagnosis of SSc (13) and 8 were classified as early SSc, both according to VEDOSS proposal and to patient-derived definitions (14-16).

The patients underwent a thorough physical examination and a number of instrumental investigations in order to assess organ involvement, according to a defined core set of variables to be used in clinical studies for SSc (18). Skin involvement was assessed using the modified Rodnan skin score (19). Oesophageal involvement was assessed by esophagogram. Pulmonary arterial hypertension was screened by echocardiogram and confirmed by right heart catheterisation. Interstitial lung disease was assessed by chest x-ray, HR CT lung scans and respiratory function tests. The presence of a history of digital ulcer was recorded as well as major cardiovascular risk factors such as smoking, diabetes, hypertension, dyslipidemia. Caffeine, nicotine and non-steroidal anti-inflammatory medications were forbidden to all the patients 24 hours prior to the test. Vasodilator medications were allowed provided the dose was stable in the 12 weeks prior to the test. Patients who underwent Iloprost treatments were assessed just before the infusion. Epidemiological and clinical data, collected at time of laser imaging, are further detailed in Table II.

Laser speckle flowmetry

Cutaneous blood flow was measured throughout the experiments using a high frame rate LASCA (Pericam PSI system, Perimed, Jarfalla). Laser wavelength was 785 nm. The laser scanning device was placed 20 cm above the skin of the dorsum of the hand. The image acquisition rate was 10 s^{-1} and frames were 11 x 11 cm. The instrument was calibrated using a suspension of polystyrene microspheres according to the manufacturer's instruction. The patients were firstly acclimatised by getting them to rest at a constant temperature (22-24°C) for 30 minutes. The basal recording lasted 5 minutes. The colour image of the dorsum of the hand was assessed to evaluate qualitative parameters, such as the presence of proximal distal gradient and homogeneity of flux distribution, as previously described (20).

Then the patients underwent both the ischaemic and cold test. Since the reproducibility of the tests has not been assessed in this study, we did not consider the raw data but expressed the flux after dynamic stimuli as the relative percentage variations with respect to the basal flux, setting the baseline at 100% (21).

The occlusive/ischaemic test was performed by inflating for 4 minutes a cuff placed on the left arm to 30 mm Hg above the systolic pressure. The recovery time (time needed to recover the basal flux after occlusion in seconds), the peak flux (hyperaemic peak reached after occlusion) and the area under the hyperaemic curve were recorded.

After 30 minutes of further acclimatisation, the cold test was performed by dipping the left hand (covered by a latex glove) in refrigerated water at 16°C for 90 seconds. The glove was then immediately removed registering the blood flow in the same hand until re-warming and recovery of the basal flux. The cold flux (the flux after the cold test) and the time of recovery (time of recovery of the basal flux expressed in minutes) were recorded.

Nailfold video-capillaroscopy

Nailfold videocapillaroscopy was performed using an optical probe-equipped videocapillaroscope with a 200 x contact lens (Scopeman, Pico charm view), connected to image analysis software (Alpha strumenti, Milan, Italy). Capillaroscopic patterns in SSc patients were classified according to Cutolo *et al*. (8). Table III shows capillaroscopic patterns and capillary density (per linear mm) in Raynaud's patients.

Statistical analysis

Data of the patients were stored in a computerised database. Statistical analysis was performed by Statview software. Demographic and clinical data were analysed by descriptive statistic. Correlation between clinical data and laser measurements were performed by Spearman's rank test, ANOVA post hoc test and logistic regression, both univariate and multivariate. Values are expressed as mean ± 1 standard deviation (SD) unless otherwise indicated. In view of the high number of comparisons involved, Bonferroni correction was applied.

Results

Basal recording

Basal recording was significantly higher in SSc patients (29.5±15) as compared to HS (19±8) and PRP subjects (15±8) (p=0.0025 and <0.0001) (Fig. 1). Basal values were weakly correlated to age (Spearman rank's correlation 0.26 p=0.02). The differences between groups were maintained when corrected for age. Since half of the patients with SSc and none of PRP and HS, were treated with vasodilator drugs, we assessed if there were differences in the basal values according to treatment, but no discrepancy was noted (29.6 with *vs*. 29.5 PU without treatment p=ns).

No statistical significant differences in the basal values were noted between patients with early disease and long-standing disease, nor according to disease subset (limited *vs*. diffuse), vasodilator therapy, capillaroscopic pattern, type of organ involvement or to the presence/absence of cardiovascular risk factors.

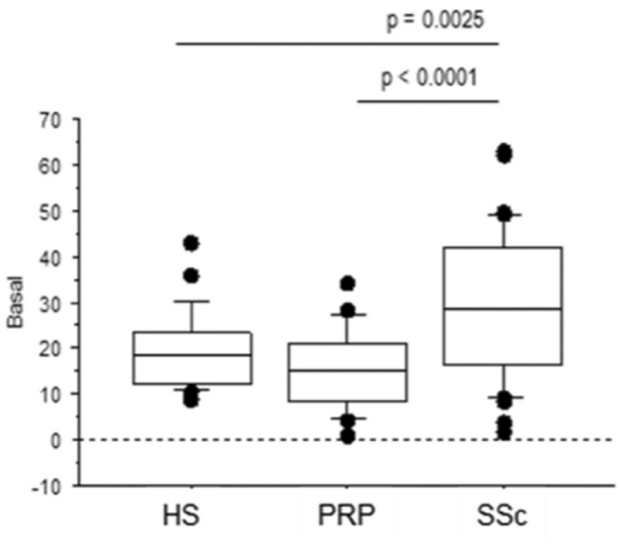


Fig. 1. Differences in basal recording between 36 SSc patients as compared to 20 HS and 20 PRP subjects (p=0.0025 and <0.0001). In the box plots the 10th, 25th, 50th (median), 75th and 90th percentile of each variable are shown.Values above the 90th centile and below the 10th centile are plotted as points.

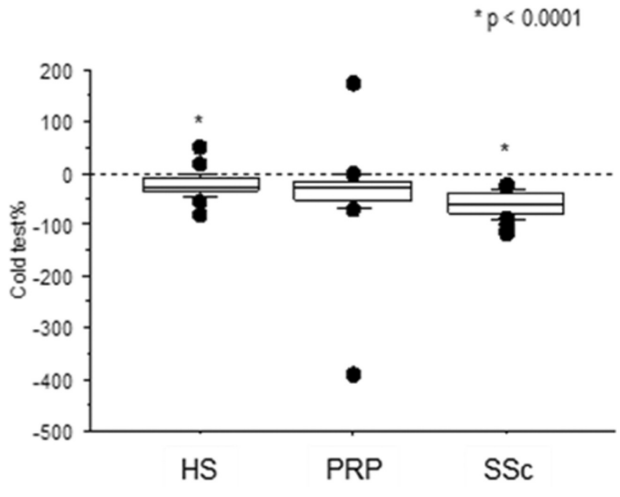


Fig. 2. Cold challenge: percent variation from the basal value of HS as compared to PRP and SSc patients (p<0.0001). In the box plots the 10th, 25th, 50th (median), 75th and 90th percentile of each variable are shown. Values above the 90th centile and below the 10th centile are plotted as points.

Qualitative parameters

The distribution of skin flow in the dorsum of the hand was significantly different between HS, PRP and SSc-RP. Indeed, proximal distal gradient was noticed in 74% of HS, 45% of PRP and 36% of SSc-RP (p=0.01). Homogeneity of flux distribution was detected in 95% of healthy subjects, 80% of PRP and 16% of SSc-RP (p<0.0001).

Cold test

Figure 2 shows the percentage variation from the baseline values during the cold test. After cold test, SSc-RP had a significant reduction in blood flow (-58±24%) as compared to HS (-19±25%) (p=0.01). The time necessary to recover the basal flow was significantly higher in SSc-RP (58±43 minutes) as compared to HS (18±29 minutes) and PRP (19±24 minutes) (p=0.006 and 0.0016, respectively).

When the SSc patients were divided according to the cutaneous subset (diffuse *vs*. limited), no statistical differences were outlined. Moreover, no significant statistical differences in the cold challenge were found between patients with early and long-standing disease, nor according to vasodilator therapy, capillaroscopic pattern, type of organ involvement or to the presence/absence of cardiovascular risk factors.

Occlusive/hyperaemia test

Following brachial artery occlusion, although the time to recover of the pre-test flux was longer in SSc patients (mean 291±611 sec) as compared to HD (179±357 sec) and PRP (170±222 sec), the difference was not statisti-

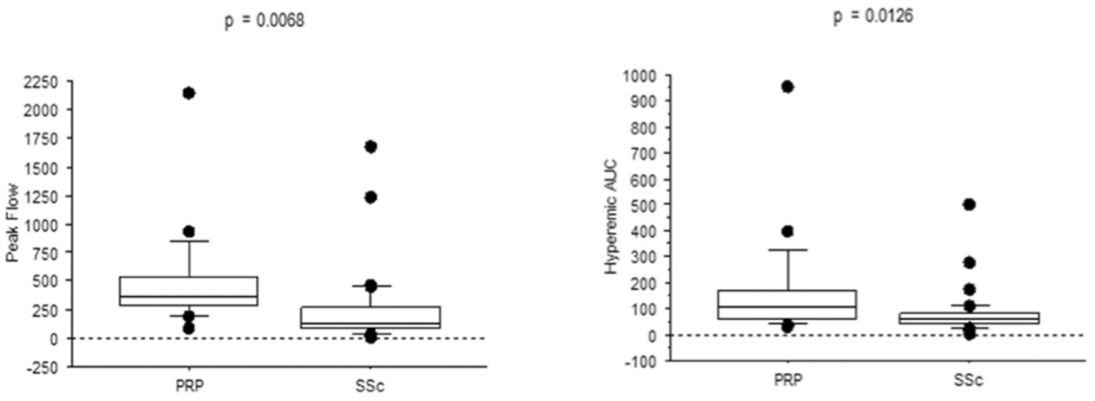


Fig. 3. Differences in post-ischaemic peak flow (p=0.0068) and in the hyperaemic area under the curve (p=0.0126) between PRP and SSc patients. In the box plots the 10th, 25th, 50th (median), 75th and 90th percentile of each variable are shown. Values above the 90th centile and below the 10th centile are plotted as points.

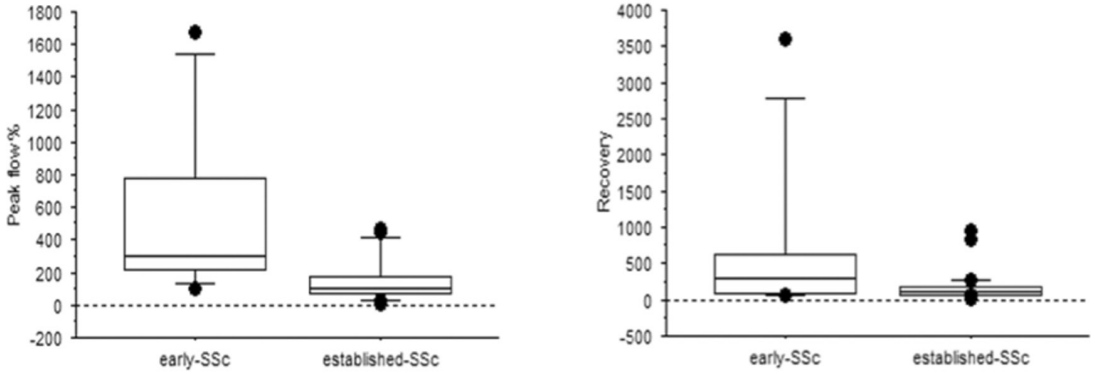


Fig. 4. Differences in post-ischaemic peak flow (p=0.0068) and in time of recovery after the ischaemic test (p=0.047) between early-SSc and established-SSc. In the box plots the 10th, 25th, 50th (median), 75th and 90th percentile of each variable are shown. Values above the 90th centile and below the 10th centile are plotted as points.

cally significant. The post-occlusion peak flow was significant reduced in SSc-RP (+237%) as compared to PRP (+485±435%) (p=0.0068). The area under the hyperaemic curve was significantly reduced between SSc-RP (79±87 U/sec) as compared to PRP (167±204 U/sec) (p=0.0126) (Fig. 3). Among patients affected by Raynaud's phenomenon, peak flow and the area under the hyperaemic curve were correlated to capillary density (Spearman rank's correlation: peak flow: 0.53 p<0.0001; area under the hyperaemic curve: 0.49 p=0.0002).

Among SSc patients, a significant difference in peak flow after ischaemic test (543±600 % *vs*. 150±135 % p=0.0068), in the post-occlusive hyperaemic area under the curve (158±163 *vs*. 58±29 U/sec p=0.005) and in duration of hyperaemic response after ischaemic test (711±1211 seconds *vs*. 171±224 seconds p=0.047) was shown between patients with early-SSc *versus* patients with definite SSc (Fig. 4).

No difference in the dynamic of microcirculation was noticed between diffuse and limited disease, nor according to vasodilator therapy, capillaroscopic pattern, type of organ involvement or to the presence/absence of cardiovascular risk factors.

Differences between groups sorted out by univariate analysis were entered in a multivariate model to outline items that independently discriminated between groups. Homogeneity of flux distribution (p<0.0001) independently discriminated between HS, PRP and SSc-RP, respectively.

Discussion

LASCA allows non-invasive qualitative and quantitative analysis of the microcirculation. Most previous studies were performed by laser Doppler and few data are available with this technique in Raynaud's phenomenon.

Studies on single point laser Doppler (LD) flowmetry have shed light on the dynamic of microcirculation in Raynaud's phenomenon. The results are not always consistent, however, due to the variability of LD technique and poor reproducibility.

Basal skin blood flow have been claimed to be either reduced or normal in SSc patients as compared to healthy people (22-23). Skin perfusion showed a correlation with the progression of vascular damage evaluated by capillaroscopy (24). Ischaemic challenge and cold test have yielded similar results, where some studies reported no consistent differences in these tests between primary and secondary RP, while others contradicted these results (22, 24). A different kinetic of response to ischaemic challenge and cold stimulus have been claimed within SSc cutaneous subset, diffuse disease showing a distinct alteration of ischaemic test and limited disease with a prevalent affection of the response to cold (25). These discrepancies might be responsible for blunting differences when dissimilar proportion of the disease subsets are mixed in different studies.

A number of surveys have been performed by laser Doppler perfusion imaging (LDI) (26-29). This technique allows the mapping of a large skin area. It is therefore possible to assess regional differences in the intensity and in the distribution of perfusion. Proximal/distal gradient and homogeneous dis-

tribution is typical of a healthy subject, while PRP subjects show homogeneous distribution without proximal/distal gradient and eventually SSc patients generally lack either proximal/distal gradient and homogeneity of flux distribution (26-27). Our study confirms these results, showing a homogeneous distribution of flux in only 16% of SSc patients, as compared to 80% of PRP and 95% of HS, while proximal/distal gradient is more frequent in HS as compared to PRP and SSc. Regional differences in the response to cold challenge have been outlined in SSc patients according to capillaroscopic progression (28). Also, the response to the administration of Bosentan shows regional variations, with a more striking increase in the skin region distal to the interphalangeal joint. The response to treatment is affected even by capillaroscopic pattern, with wider improvements in subjects with early and active pattern as compared to late pattern (29). Local differences in the distribution and intensity of flow might predict the development of ischaemic digital lesions (30).

The main limitation of LDI, however, is the long time needed for scanning, that may not allow the detection of quick variations during dynamic tests.

According to these experiences, there is no uniform approach in the assessment of microvascular involvement in Raynaud's phenomenon. The most reliable information could probably be obtained by a combination of different techniques, including capillaroscopy and thermography (31).

In this preliminary report we have demonstrated that LASCA is able to pick out a number of differences between the HS and RP population, particularly in the subset of SSc-RP. We detected a significant increase in basal flow in SSc patients as compared to HS and PRP patients. We have no clear explanation for this phenomenon; a significant number of our SSc patients (50%) were under vasodilator treatment at time of study, all of the treated patients were taking calcium channel blockers, while six of them received monthly infusions of Iloprost. When patients were divided according to therapy, however, no significant difference emerged. Despite having higher values in basal condition, SSc patients showed a marked depression of flow under cold challenge as compared to HS. The time of rewarming was slower both in comparison to HS and PRP patients.

As regards the post-occlusive test, SSc patients showed a blunted response, both in terms of peak flow and in the hyperaemic area under the curve, as compared to PRP.

When SSc patients were stratified according to early and established SSc, a peculiar difference in the ischaemic challenge was noticed. Early-SSc patients showed a significant increase in the amplitude of the hyperaemic response (both in peak flow and the area under the hyperaemic curve), but also a prolongation of the recovery time as compared to established SSc. This empirical observation is consistent with the detection of high level of proangiogenic cytokines observed in the earliest stages of the disease as a compensatory phenomenon against ischaemia (32). Overexpression of these cytokines, however, is insufficient to trigger the repair process and the persistent upregulation might rather deteriorate vascular processes than support the formation of new vessels (33-35).

Our preliminary results indicate a clearcut alteration of the dynamic of microcirculation in SSc-RP as compared to PRP and HS. Among SSc patients, early disease seems to have a different pattern of microvascular reactivity as compared to established disease. The transition of microvascular alterations from the beginning of the disease to the late stages encompass a number of steps that have been comprehensively described by capillaroscopy. In the initial stages, the hallmark of microvascular involvement is the presence of giant capillaries without architectural derangement, while the following steps are characterised by a progressive loss of capillaries, that mirrors the loss of vasculogenetic and angiogenetic capacity, well described by Cutolo *et al*. (8). In this study we described for the first time the functional alterations in early SSc as compared to established disease and noticed that a peculiar alteration of the ischaemic test is involved in the earliest stages of the disease. This peculiar alteration of microvascular reactivity lends support to the hypothesis that the disease starts with the beginning of Raynaud's phenomenon rather than with the appearance of the first non-Raynaud's symptom (17). Moreover, it strengthens the concept that an early detection of a SSc pattern on capillaroscopy should compel our diagnosis on SSc spectrum rather than on undifferentiated connective tissue disease (36-40). Further validation of these results on a larger series of patients are under way.

In a clinical setting, LASCA may prove useful for better refining the discrimination between primary and secondary Raynaud's phenomenon and possibly characterising SSc microangiopathy. Moreover, the advantage of LASCA is that combines the rapidity of acquisition with the possibility of mapping a relatively large skin area. These features may allow the study of regional differences in skin perfusion that may have prognostic significance and to evaluate *in vivo* and in real time the effect of pharmacological challenge on selected areas in order to avoid useless and potentially harmful administration of expensive vasodilator treatments, in an era where patient centered care and cost/effectiveness of medications have to be taken into account (41). On the other hand, the main limitations of LASCA are the lack of spectral information and of absolute quantitative measurements (42).

References

1. MOSCA M, TANI C, NERI C, BALDINI C, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD). *Autoimmun Rev* 2006; 6: 1-4.
2. TANI C, MOSCA M, D'ASCANIO A *et al*.: Chronic inflammation and endothelial dysfunction: analysis of a cohort of patients with SLE and UCTD. *Reumatismo* 2006; 58: 212-8.
3. MOSCA M, BALDINI C, BOMBARDIERI S: Undifferentiated connective tissue diseases in 2004. *Clin Exp Rheumatol* 2004; 22 (Suppl. 33): S14-8.
4. MOSCA M, NERI R, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD): a review of the literature and a proposal for preliminary classification criteria. *Clin Exp Rheumatol* 1999; 17: 615-20.
5. CAPPELLI S, BELLANDO RANDONE S,

MARTINOVIĆ D *et al*.: "To be or not to be," ten years after: evidence for mixed connective tissue disease as a distinct entity. *Semin Arthritis Rheum* 2012; 41: 589-98.
6. KUMAR P, GALARRAGA B AND BELCH J: Raynaud's phenomenon. Section 10. Connective tissue disorders. *In* HOCHBERG MC (Ed.): Rheumatology. Mosby 2011: 1427.
7. MARICQ HR, HARPER FE, KHAN MM, TAN EM, LEROY EC: Microvascular abnormalities as possible predictors of disease subsets in Raynaud phenomenon and early connective tissue disease. *Clin Exp Rheumatol* 1983; 1: 195-205.
8. CUTOLO M, PIZZORNI C, TUCCIO M *et al*.: Nailfold videocapillaroscopic patterns and serum autoantibodies in systemic sclerosis. *Rheumatology* 2004; 43: 719-26.
9. INGEGNOLI F, BORACCHI P, GUALTIEROTTI R *et al*.: Prognostic model based on nailfold capillaroscopy for identifying Raynaud's phenomenon patients at high risk for the development of a sclerodermia spectrum disorder. *Arthritis Rheum* 2008; 58: 2174-82.
10. LEROY EC: Systemic sclerosis. A vascular perspective. *Rheum Dis Clin North Am* 1996; 22: 675-94.
11. ROUSTIT M, MILLET C, BLAISE S, DOUFORNET B, CRACOWSKY JL: Excellent reproducibility of laser speckle contrast imaging to assess skin microvascular reactivity. *Microvasc Res* 2010 80: 505-11.
12. LEROY EC, MEDSGER TA: Rayanud's phenomenon: a proposal for classification. *Clin Exp Rheumatol* 1992; 10: 485-8.
13. MASI AT, RODNAN GP, MEDSGER TA JR. *et al*.: Preliminary criteria for the classification of systemic sclerosis (scleroderma). *Arthritis Rheum* 1980; 23: 581-90.
14. AVOUAC J, FRANSEN J, WALKER UA *et al*.: Preliminary criteria for the very early diagnosis of systemic sclerosis: results of a Delphi Consensus Study from EULAR Scleroderma Trials and Research Group. *Ann Rheum Dis* 2011; 70: 476-81.
15. KOENIG M, JOYAL F, FRITZLER MJ *et al*.: Autoantibodies and microvascular damage are independent predictor factors for the progression of Raynaud's phenomenon to Systemic sclerosis. *Arthritis Rheum* 2008; 58: 3902-12.
16. VALENTINI G, CUOMO G, ABIGNANO G *et al*.: Early systemic sclerosis: assessment of clinical and pre-clinical organ involvement in patients with different disease features. *Rheumatology* 2011; 50: 317-23.
17. VALENTINI G, VETTORI S, CUOMO G *et al*.: Early systemic sclerosis: short term disease evolution and factors predicting the development of new disease manifestations of organ involvement. *Arthritis Res Ther* 2012; 14: R188.
18. VALENTINI G: Appendix. Manual of signs, symptoms, methods and procedures for the assessment of the patients with SSc. *Clin Exp Rheumatol* 2003; 21 (Suppl. 23): 49-56.
19. CLEMENTS P, LACHENBRUCH P, SEIBOLD J *et al*.: Inter- and intra-observer variability of the total thickness score (modified Rodnan TSS) in systemic sclerosis. *J Rheumatol* 1995; 22: 1281-5.
20. ROSATO E, GIGANTE A, BARBANO B *et al*.: In systemic sclerosis macrovascular damage of hands digital arteries correlates with microvascular damage. *Microvasc Res* 2011; 82: 410-5.
21. ROUSTIT M, MAGGI F, ISNARD S, HELLMANN M, BAKKEN B, CRACOWSKI JL: Reproducibility of a local cooling test to assess microvascular function in human skin. *Microvasc Res* 2010; 79: 34-9.
22. CORREA MJU, ANDRADE LEC, KAYSER C: Comparison of laser doppler imaging, fingertip lacticemy test and nailfold capillaroscopy for assessment of digital microcirculation in systemic sclerosis. *Arthritis Res Ther* 2010; 12: R157.
23. CUTOLO M, FERRONE C, PIZZORNI C, SOLDANO S, SERIOLO B, SULLI A: Peripheral blood perfusion correlates with microvascular abnormalities in systemic sclerosis: a laser-Doppler and nailfold videocapillaroscopy study. *J Rheumatol* 2010; 37: 1174-80.
24. RAJAGOPALAN S, PFENNINGER D, KEHRER C *et al*.: Increased Asymmetric Dimethylarginine and Endothelin 1 Levels in Secondary Raynaud's Phenomenon Implications for Vascular Dysfunction and Progression of Disease. *Arthritis Rheum* 2003; 48: 1992-2000.
25. GRATTAGLIANO V, IANNONE F, PRAINO E *et al*.: Digital laser doppler flowmetry may discriminate "limited" from "diffuse" systemic sclerosis. *Microvasc Res* 2010; 80: 221-6.
26. ROSATO E, BORGHESE F, PISARRI S, SALSANO F: Laser Doppler perfusion imaging is useful in the study of Raynaud's phenomenon and improves the capillaroscopic diagnosis. *J Rheumatol* 2009; 36: 2257-63.
27. ROSATO E, MOLINARO I, ROSSI C, PISARRI S, SALSANO F: The combination of laser Doppler perfusion imaging and photoplethysmography is useful in the characterization of scleroderma and primary Raynaud's phenomenon. *Scand J Rheumatol* 2011; 40: 292-8.
28. ROSATO E, ROSSI C, MOLINARO I, GIOVANNETTI A, PISARRI S, SALSANO F: Laser Doppler perfusion imaging in systemic sclerosis impaired response to cold stimulation involves digits and hand dorsum. *Rheumatology* 2011; 50: 1654-8.
29. ROSATO E, MOLINARO I, BORGHESE F, ROSSI C, PISARRI S, SALSANO F: Bosentan improves skin perfusion of hands in patients with systemic sclerosis with pulmonary arterial hypertension. *J Rheumatol* 2010; 37: 2531-9.
30. ROSATO E, ROUMPEDAKI E, PISARRI S, SALSANO F: Digital ischemic necrosis in a patient with systemic sclerosis: the role of laser Doppler perfusion imaging. *Vasa* 2009; 38: 390-3.
31. MURRAY AK, MOORE TL, MANNING GB, TAYLOR C, GRIFFITHS CEM, HERRICK A: Non-invasive imaging techniques in the assessment of scleroderma spectrum disorders. *Arthritis Rheum* 2009; 61: 1103-11.
32. DISTLER O, DEL ROSSO A, GIACOMELLI R *et al*.: Angiogenic and angiostatic factors in systemic sclerosis: increased levels of vascular endothelial growth factor are a feature of the earliest disease stages and are associated with the absence of fingertip ulcers. *Arthritis Res* 2002; 4: R11.
33. CIPRIANI P, MARRELLI A, LIAKOULI V, DI BENEDETTO P, GIACOMELLI R: Cellular players in angiogenesis during the course of systemic sclerosis. *Autoimmun Rev* 2011; 10: 641-6.
34. DISTLER O, DISTLER JH, SCHEID A *et al*.: Uncontrolled expression of vascular endothelial growth factor and its receptor leads to insufficient skin angiogenesis in patients with systemic sclerosis. *Circ Res* 2004; 95: 109-16.
35. RICCIERI V, STEFANANTONI K, VASILE M *et al*.: Abnormal plasma levels of different angiogenic molecules are associated with different clinical manifestations in patients with systemic sclerosis. *Clin Exp Rheumatol* 2011; 29 (Suppl. 65): S46-S52.
36. MOSCA M, TANI C, CARLI L, BOMBARDIERI S: Undifferentiated CTD: a wide spectrum of autoimmune diseases. *Best Pract Res Clin Rheumatol* 2012; 26: 73-7.
37. MOSCA M, TANI C, TALARICO R, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD): simplified systemic autoimmune diseases. *Autoimmun Rev* 2011; 10: 256-8.
38. MOSCA M, VIRDIS A, TANI C *et al*.: Vascular reactivity in patients with undifferentiated connective tissue diseases. *Atherosclerosis* 2009; 203: 185-91.
39. MOSCA M, TANI C, NERI C *et al*.: Analysis of the evolution to defined connective tissue diseases of patients with "early unidifferentiated connective tissue diseases (UCTD)". *Reumatismo* 2008; 60: 35-40 (Italian).
40. MOSCA M, TANI C, BOMBARDIERI S: Undifferentiated connective tissue diseases (UCTD): a new frontier for rheumatology. *Best Pract Res Clin Rheumatol* 2007; 21: 1011-23.
41. THOMBS BD, JEWETT LR, ASSASSI S *et al*.: New directions for patient-centred care in scleroderma: the Scleroderma Patient-centred Intervention Network (SPIN). *Clin Exp Rheumatol* 2012; 30 (Suppl. 71): S23-S29.
42. CUTOLO M: Techniques for evalutating the microcirculation. Atlas of Capillaroscopy in Rheumatic diseases, Elsevier. 2010; 3: 17-31.