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Correlation between circulating fibrocytes and dermal thickness in limited cutaneous systemic sclerosis patients: a pilot study

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Abstract

The objective is to detect any possible correlation between the modified Rodnan skin score (mRSS) and dermal thickness (DT) measured by skin high-frequency ultrasound (US) and the percentage of circulating fibrocytes in patients with limited cutaneous systemic sclerosis (lcSSc). Eight lcSSc patients and five healthy subjects (control group, CNT) were enrolled. The skin involvement was evaluated by mRSS and US (18 and 22 MHz probes) in all 13 subjects in the 17 standard skin areas evaluated by mRss. Circulating fibrocytes were isolated from the peripheral blood mononuclear cells (PBMCs) of all lcSSc patients and the CNT group to analyze their percentage at baseline time (T0) when the experiments started with PBMCs' isolation and collection and after 8 days of culture (T8). Non-parametric tests were used for the statistical analysis. A positive correlation between the percentage of circulating fibrocytes at T0, mRSS ($p=0.04$ $r=0.96$), and DT-US, evaluated by the 22 MHz and the 18 MHz probes ($p=0.03$, $r=0.66$ and $p=0.05$, $r=0.52$, respectively), was observed in lcSSc patients. Conversely, at T8, there was no correlation ($p>0.05$) between these parameters in lcSSc group. In the CNT group, no correlations between mRSS or DT-US and the percentage of circulating fibrocytes were observed both at T0 and T8. The study shows the presence of a significant relationship between the percentage of circulating fibrocytes and DT, as evidenced by both mRSS and US, in limited cutaneus SSc. This observation may well suggest the reasonable hypothesis of a crucial contribution of circulating fibrocytes to skin fibrosis progression, which might be considered as further biomarkers.

Keywords Systemic sclerosis · Modified Rodnan skin score · High-frequency ultrasound · Fibrocytes

Introduction

Systemic sclerosis (SSc) is a complex autoimmune connective tissue disease characterized by a chronic and progressive tissue and organ involvement, including skin and lung [1–4]. Skin damage is a marker for disease classification and activity [1–4].

SSc is sub-classified into limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc), based on the extent of skin involvement. In patients with lcSSc, skin involvement is distal to elbows and knees, with or without involvement of face; dSSc is characterized by the presence of skin thickening also on the upper arms, chest, abdomen, and thighs [1].

The modified Rodnan skin score (mRSS) is the validated method to measure skin thickness and is used in clinical trials to evaluate the severity of skin damage [1, 4–7]. As mRSS has a high intra- and inter-observer variability [5–7], recent research has been focused on the utility of high-frequency ultrasound (US) for an early identification of skin involvement in SSc patients [7–12].

Fibrosis in SSc is due to the activation of fibroblasts (from different sources) and their transition into profibrotic myofibroblasts [13–19]. Indeed, after their phenotype transition, myofibroblasts acquire an increased persistent capability to synthesize and accumulate extracellular matrix (ECM) proteins, such as type I collagen (COL-1) and fibronectin (FN), initiating an altered process leading to a systemic fibrosis [13–19]. Myofibroblasts may originate from the transition of

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several other cell types, including endothelial and epithelial cells, pericytes, and circulating fibrocytes [20–22].

Fibrocytes are a subpopulation of progenitor cells derived from bone marrow, characterized by the expression of markers belonging to leukocytes and haematopoietic cells, such as CD45, major histocompatibility complex class-II (i.e., HLA-DR) and CD34, as well as stromal cell markers, primarily type I collagen [23]. Moreover, fibrocytes express chemokine receptors, such as CCR2 and CXCR4 that are essential for circulating fibrocytes migration into inflammatory lesions and/or damaged tissues and they allow the differentiation of these cells into myofibroblasts [24]. The ability to differentiate into fibroblasts/myofibroblasts is fundamental in physiological conditions, including wound healing, whereas it has been demonstrated that in chronic fibroproliferative disorders, circulating fibrocytes are recruited at the level of damaged tissues though CXCR4/CXCL12 interaction, where they differentiate into fibroblasts–myofibroblasts, secreting essential ECM proteins, primarily type I collagen and fibronectin, and contributing to fibrosis [19, 25–28].

It has been demonstrated that in chronic fibroproliferative disorders, circulating fibrocytes are recruited at the level of damaged tissues though CXCR4/CXCL12 interaction, where they differentiate into fibroblasts–myofibroblasts, secreating essential ECM proteins and contributing to fibrosis [19, 25–28]. Myofibroblast transition and ECM overproduction are known to be induced by several profibrotic mediators, including transforming growth factor-β1 (TGFβ1), endothelin-1 (ET-1), cytokines, and chemokines (such as IL-6 and CCL18), whose circulating levels are increased in patients with SSc [13–19, 29, 30].

Therefore, the transition of fibrocytes may represent an important mechanism responsible for the accumulation of profibrotic myofibroblasts at the level of damaged tissues, such as the skin and lung, in several fibrotic diseases, including SSc and pulmonary fibrosis [18, 19, 31].

Based on this knowledge, the blockage of the transition into myofibroblasts of several cell types including fibroblasts and circulating fibrocytes, together with the reduction of the profibrotic myofibroblast activity and related ECM overproduction, might represent important steps in contrasting the fibrotic process at least in SSc [13–20].

The aims of this study were:

(i) to evaluate any correlation between DT, measured by both mRSS and US (18 and 22 MHz probes) and the percentage of circulating fibrocytes in lcSSc patients;

(ii) to confirm any correlation between US and mRSS in the evaluation of skin involvement in lcSSc patients.

Methods

Study population

After obtaining the local Ethics Committee approval and written informed consent, 8 lcSSc patients (7 females and 1 male) and 5 (4 females and 1 male) age-matched healthy volunteers (CNT) were enrolled. The lcSSc patients were enrolled during routine clinical follow-up and they all fulfilled the 2013 ACR/EULAR criteria for SSc [3]. A complete medical history was taken for all patients and a clinical examination was performed, as reported in Table 1. The results of the mRSS were equal to zero at the level of the upper arms, chest, abdomen, and thighs, according to the lcSSc classification [1, 4–6].

As no evident clinical SSc complications were present other than skin involvement and Raynaud's phemomenon, the lcSSc patients were on vasodilators (mainly cyclic prostanoids) at enrollment. All instrumental examinations (i.e., pulmonary function testing, chest CT, echocardiography, etc.) were normal (Table 1).

The modified Rodnan skin score (mRSS)

The skin involvement was evaluated by mRSS in all 13 subjects in the 17 standard skin areas evaluated by mRss (zygoma, fingers, dorsum of the hands, forearms, upper arms, chest, abdomen, thighs, legs, and feet) [4, 8, 9]. Their skin thickness was assessed by skin palpation and graded on a scale from 0 to 3, where 0 = normal, 1 = weak, 2 = intermediate, and 3 = severe skin thickening [4, 8, 9].

As aforementioned, only patients classified as having lcSSc were enrolled into the study to investigate any correlation between the mRSS and DT values (measured by both 18 and 22 MHz probe) in lcSSc patients and to compare these results to the CNT. The mRSS was equal to zero in all skin areas of the CNT subjects. The same operator performed the mRSS in all subjects and was blinded to the US assessment.

Skin high-frequency ultrasound (US)

Two different US transducers were used in all the 17 skin areas of patients and CNT: an 18 MHz (MyLab 25, Esaote, Genoa, Italy) and a 22 MHz (MyLab One, Esaote, Genoa, Italy). The US measurements were based on two-bidimensional B-mode images. As reported in preview studies, an electronic calibre was used to measure the dermal thickness (DT) [9, 11]. The ultrasound values were recorded in millimetres. Both mRSS and US were performed on the same day in all patients.

Table 1 Clinical findings in in limited cutaneous systemic sclerosis (lcSSc) and healthy subjects (CNT)

	CNT (#5) median [IQR]	lcSSc (#8) median [IQR]	lcSSc vs. CNT Stat. signif.
Age (years)	60.0 [3.2]	60.0 [10.7]	$p > 0.05$
RP duration (years)	–	15.5 [11.7]	NA
SSc duration (years)	–	7.8 [8.1]	NA
mRSS Total (score)	–	17.5 [4.8]	NA
Organ involvement	–	DU1, GI 0, Lung 0, Heart 0	NA
Skin involvement	–	lcSSc 8 dcSSc 0	NA
ANA patterns	–	Centrom 7 Speckled 1	NA
Capillaroscopy pattern	–	Early 0, Active 8 Late 0	NA
Treatments	PPI 2	Vasodilat 8, Aspirin 7 PPI 7	NA

The results were reported as median and interquartile range [IQR]

RP Raynaud's phenomenon, *mRSS* modified Rodnan skin score, *DU* digital ulcer presence at the evaluation, *GI* gastrointestinal involvement in our patients was represented by alteration of gastrointestinal motility evaluated by manometry, *Lung* lung involvement in our group of SSc was represented by mild/moderate fibrosis at the HRCT evaluation, *Heart* cardiac involvement was rapresented by presence of systolic pulmonary artery pressure > 40 mmHg at echocardiography; limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc), antinuclear antibody (ANA) patterns (indirect immunofluorescence on HEp-2 cells), centrom: centromeric (ACA +), early, active, *late* patterns of microangiopathy at nailfold videocapillaroscopy, *vasodilat* vasodilators, *PPI* proton pump inhibitor. *NA* not applicable

Nailfold videocapillaroscopy (NVC)

NVC was performed by an optical probe, equipped with a 200× contact lens, connected to image analysis software (Videocap, DS Medica, Milan, Italy), to select only patients with an "Active" microangiopathy pattern, according to the classification by Cutolo et al. [32–34]. We decided to enroll only patients with an "Active" microangiopathy pattern to obtain an intermediate microvascular damage, as those with a "Late" pattern are more likely to have organ damage, to avoid any bias due to the need for immunomodulators [9–12, 35].

The same operator performed the NVC evaluations and was blinded to the skin assessment.

Cell culture

Circulating fibrocytes were obtained from all the lcSSc and CNT subjects enrolled. Fibrocytes were isolated from peripheral blood mononuclear cells (PBMCs) and characterized by fluorescence-activated cell sorter analysis (FACS) at baseline (T0) and after 8 days of culture (T8) in Dulbecco's Modified Eagle's Medium (DMEM, Euroclone, Milan, Italy) at 20% of Fetal Bovine Serum using anti-CD45, CXCR4, CD14, HLA-DRII (Beckman Culter, CA, USA), and anti-COL-1 (Millipore, MA, USA) conjugated primary antibodies in line with several other studies [18, 19].

The experiments on circulating fibrocytes obtained from each lcSSc patient and voluntary healthy subject were done in triplicate

Quantitative real-time polymerase chain reaction (qRT-PCR) was performed for COL-1, TGFβ1, and the myofibroblast phenotype markers α-smooth muscle actin (αSMA), fibroblast specific protein-1 (S100A4), and β-actin (housekeeping gene) using specific primers supplied by Primer Design, on T8-cultured SSc and CNT fibrocytes [19, 20]. To obtain the gene expression values, for each lcSSc patients and CNT group, the mean Ct value of the target gene was normalized with that of the related housekeeping gene in circulating fibrocytes. The resulting value of the target gene from circulating fibrocytes of each enrolled SSc patients was normalized with that of CNT group (which represents the calibrator used for the relative quantification, in accordance with the ΔΔCt method), to obtain the gene expression level [36].

Statistical analysis

The statistical processing of the collected data was carried out by non-parametric tests: a Mann–Whitney *U* test was performed to compare unpaired groups of variables and a Kruskal–Wallis test to compare continuous variables with nominal variables with more than two levels. The Spearman rank correlation test was used to search for any relationships between variables, along with linear regression tests. The

Intraclass Correlation Coefficient (ICC) was calculated to assess intra-operator repeatability. Any p value lower than 0.05 was considered statistically significant. The results are reported as median and interquartile range (IQR).

Results

There was a positive correlation in lcSSc patients between the percentage of circulating fibrocytes at T0 and both mRSS ($p=0.04$, $r=0.96$) and DT, evaluated by the two probes ($p=0.05$, $r=0.52$ and $p=0.03$, $r=0.66$ for the 18 and 22 MHz probes, respectively) (Table 3).

No correlations were observed between the T8-cultured fibrocytes, mRSS or US (the two probes) in the lcSSc patients. Moreover, there was no correlation, between the aforementioned values (mRSS vs. fibrocytes $p>0.05$; US 18 MHz probe vs. fibrocytes $p>0.05$; US 22 MHz probe vs. fibrocytes $p>0.05$) in CNT at either T0 or T8 (Table 3).

The FACS analysis showed an approximately twofold higher percentage of fibrocyte, at T0, which were characterized as $CD45^{+}COL\text{-}1^{+}CXCR4^{+}$ cells, in SSc patients than in CNT group (Table 2) [19]. Furthermore, there was a higher percentage of fibrocytes in both the SSc and the CNT group from T0 to T8, confirmed our previous results (Table 2) [19]. T8-cultured SSc fibrocytes were characterized also by a significant increase in basal expression of αSMA, COL-1, S100A4, and TGFβ1, compared to T8-cultured CNT fibrocytes ($p<0.01$) (Fig. 1), without any correlation between these data and the mRSS or the DT (for both probes) ($p>0.05$) (Table 3). A statistically significant correlation was observed between the mRSS and the total DT, evaluated by both probes (22 MHz $p=0.05$ $r=0.43$; 18 MHz probe $p=0.05$ $r=0.59$) and between the two transducers when evaluating DT ($p=0.04$ $r=0.60$) (Table 3). There was no correlation between the DT-US, mRSS, disease, or Raynaud's phenomenon duration ($p>0.05$). The Intraclass Correlation Coefficient (ICC) was calculated to assess intra-operator repeatability of the two probes It was 96% (95% CI 0.93–0.98) for the 18 MHz transducer, 97% for the 22 MHz (95% CI 0.96–0.98), and 95% for mRSS (95% CI 0.94–0.97).

As expected, the DT values evaluated by both probes were significantly higher in lcSSc patients than in the CNT group (Table 1).

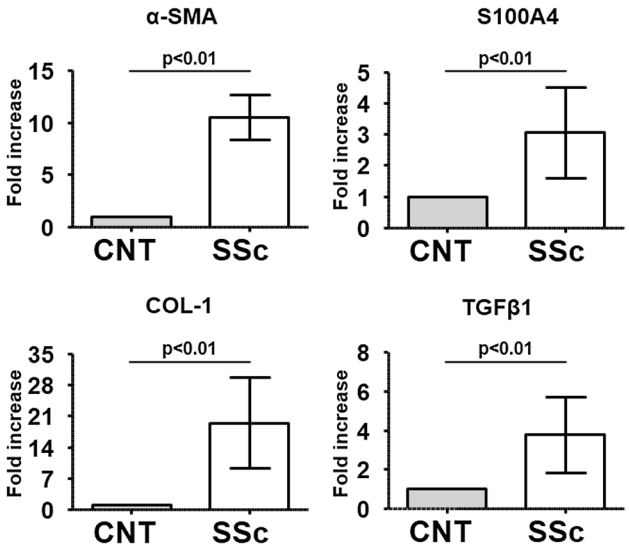

Fig. 1 Evaluation by quantitative real-time polymerase chain reaction (qRT-PCR) of the gene expression of α-smooth muscle actin (α-SMA), fibroblast specific protein-1 (S100A4), type I collagen (COL-1) and transforming growth factor-β1 (TGFβ1) in cultured human fibrocytes isolated from healthy subjects (CNT) and systemic sclerosis patients (SSc) maintained in Dulbecco's Modified Eagle's Medium (DMEM) growth medium at 20% of Fetal Bovine Serum (FBS) for 8 days (T8). The gene expression of α-SMA, S100A4, COL-1 and TGFβ1 in cultured human SSc fibrocytes was compared to that detected in cultured CNT fibrocytes, which was taken as unit value by definition

Table 2 Dermal thickness (DT, ultrasound evaluation), the percentage of fibrocytes at basal time (T0) and after 8 days of culture (T8) and gene expression values of α-smooth muscle actin (αSMA), type I collagen (COL 1), transforming growth factor-β1 (TGFβ1), and fibroblast specific protein-1 (S100A4) in limited cutaneous systemic sclerosis (lcSSc) patients and healthy subjects (CNT)

	CNT (#5) median [IQR]	lcSSc (#8) median [IQR]	lcSSc vs. CNT Stat. signif.
DT Total 18 MHz Probe (millimetres)	13.5 [1.6]	19.5 [4.1]	$p<0.01$
DT Total 22 MHz Probe (millimetres)	14.1 [1.3]	18.6 [2.7]	$p<0.01$
Fibrocytes at T0 (%)	0.4 [1.2]	0.8 [2.7]	$p>0.05$
Fibrocytes at T8 (%)	60.2 [44.6]	56.7 [40.7]	$p>0.05$
αSMA expression level (T8)	1	11.1 [6.8]	$p<0.01$
COL 1 expression level (T8)	1	22.9 [18.1]	$p<0.01$
TGFβ1 expression level (T8)	1	3.6 [3.5]	$p<0.01$
S100A4 expression level (T8)	1	2.5 [1.3]	$p<0.01$

Table 3 Correlation between the different methods (modified Rodnan skin score, high-frequency skin ultrasound with the 18 and 22 MHz probes) to evaluate dermal thickness (DT) and the percentage of fibrocytes at basal time (T0) and after 8 days of culture (T8), as well as gene expression values of α-smooth muscle actin (αSMA), type I collagen (COL 1), transforming growth factor-β1 (TGFβ1), and fibroblast specific protein-1 (S100A4) at T8 in limited cutaneous systemic sclerosis (lcSSc) patients

	mRSS total (score)	DT total 18 MHz probe (millimetres)	DT total 22 MHz probe (millimetres)
mRSS Total (score)	–	**$p=0.05$ [$r=0.59$]**	**$p=0.05$ [$r=0.43$]**
DT Total 18 MHz Probe (millimetres)	**$p=0.05$ [$r=0.59$]**	–	**$p=0.04$ [$r=0.60$]**
DT Total 22 MHz Probe (millimetres)	**$p=0.05$ [$r=0.43$]**	**$p=0.04$ [$r=0.60$]**	–
Fibrocytes at T0 (%)	**$p=0.04$ [$r=0.96$]**	**$p=0.05$ [$r=0.52$]**	**$p=0.03$ [$r=0.66$]**
Fibrocytes at T8 (%)	$p=0.9$ [$r=0.63$]	$p=0.19$ [$r=0.46$]	$p=0.92$ [$r=0.20$]
αSMA expression level (T8)	$p=0.22$ [$r=0.48$]	$p=0.48$ [$r=0.30$]	$p=0.67$ [$r=0.16$]
COL 1 expression level (T8)	$p=0.18$ [$r=0.41$]	$p=0.84$ [$r=0.29$]	$p=0.18$ [$r=44.6$]
TGFβ1 expression level (T8)	$p=0.48$ [$r=0.49$]	$p=0.94$ [$r=0.30$]	$p=0.92$ [$r=0.71$]
S100A4 expression level (T8)	$p=0.49$ [$r=0.63$]	$p=0.18$ [$r=0.45$]	$p=0.26$ [$r=0.22$]

r = correlation coefficient that measures the strength and direction of a linear relationship between two variables on a scatterplot. The r value is always between + 1 and – 1

Discussion

To the best of our knowledge, this is the first study to demonstrate a correlation between DT, evaluated by both mRSS and US and the percentage of circulating fibrocytes in lcSSc patients. Furthermore, the results confirm that the percentage of circulating fibrocytes, characterized as $CD45^+COL\text{-}1^+CXCR4^+$ cells, was higher in the SSc patients than in the CNT group [19]. Moreover, the higher expression of αSMA, COL-1, and TGFβ1 gene expression in cultured SSc compared to CNT fibrocytes suggests that they have a propensity for transition into profibrotic activated myofibroblasts, which are key cells involved in both tissue repair and fibrosis [19].

Interestingly, the two patients who had the highest increase in the percentage of circulating fibrocytes from T0 to T8 also had elevated levels in the αSMA and TGFβ1 gene expression. Furthermore, these two patients also had high US and mRSS values.

Several studies reported that circulating fibrocytes might represent a further important source of activated fibroblasts/myofibroblasts and that they might contribute to the increased presence of these cells in the tissues of SSc patients [19, 37, 38]. The important contribution of circulating fibrocytes in the early stage of dermal repair and fibrosis has also been validated by the detection of $CD45^+$COL-1-producing cells in wounded skin compared to non-wounded skin [19, 37–39].

In addition to this observation, circulating fibrocytes characterized as $CD14^-CD34^+CD45^+CXCR4^+COL\text{-}1^+$cells have been reported to be involved in the ischemic and fibrotic processes of SSc [37–39]. Indeed, it has been demonstrated that the percentage of these cells is directly correlated with the worsening of idiophatic pulmonary fibrosis and prognosis [40–42].

Moreover, in vitro and in vivo experiments have confirmed that the inhibition of fibrocyte recruitment and/or the blockage of their differentiation from precursors may limit the development of lung fibrosis [41, 42]. Circulating fibrocytes derive from bone marrow progenitor cells and in response to different stimuli, i.e., chemokine ligand–receptor pairs, they extravasate into sites of tissue injury, differentiate into fibroblasts/myofibroblasts and contribute to the generation of ECM during fibroproliferation [43–45].

Fibrocytes can be considered effector cells that exert different effects during the course of various autoimmune rheumatic diseases, such as rheumatoid arthritis (RA) and systemic lupus erythematosus (SLE) [42–46]. These data demonstrated that fibrocytes are potentially involved in disease initiation and progression [42–46]. Persistent activation of autoreactive T cells in autoimmunity may drive fibrocyte differentiation, potentially amplifying disease severity and progression [42–46]. Based on all these observations, knowing the various factors that contribute to, or antagonize, fibrocyte production and effector functions will provide potential targets for therapeutic intervention strategies in different autoimmune conditions.

The correlation observed in our study is in agreement with other studies, where fibrocytes are correlated with different tissue impairment in complex autoimmune diseases and might well also confirm the pivotal role of these cells in the process of fibrosis [42–46].

However, larger multicentre studies should be carried out to confirm these preliminary data, as a relatively small number of subjects were enrolled from a single centre.

Another limit of our manuscript was that we did not perform a cytokine profile of the supernatant from cultured

fibrocytes to understand their possible functional activity as profibrotic mediator.

Moreover, this is a cross-sectional study, while a longitudinal study would be beneficial. Therefore, the authors welcome such studies to explore the correlation they observed with disease activity. Despite these drawbacks, the observation that the percentage of circulating fibrocytes is correlated with DT-US and mRSS does carry some weight as skin involvement in SSc is not only critical for the initial diagnosis, but also has a prognostic relevance.

Further studies are ongoing, as US evaluation is able to identify the edematous phase that precedes palpable skin involvement in the early SSc stage. Indeed, adding the percentage of circulating fibrocytes to the physical evaluation (mRSS and US) might well enhance sensitivity of the early SSc diagnosis.

In conclusion, this study has evidenced an association between the percentage of circulating fibrocytes and the dermal thickness observed in lcSSc. Furthermore, the results confirm that the percentage of circulating fibrocytes was higher in the SSc patients than in the CNT group.

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Author contributions All authors have: (1) made substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; (2) been involved in drafting the manuscript or revising it critically for important intellectual content; (3) given final approval of the version to be published; and (4) agreed to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

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Compliance with ethical standards

Conflict of interest The authors declare no competing interests with this work.

Ethical approval Study was approved by Ethics Committee of the IRCCS San Martino Polyclinic Hospital of Genoa (Protocol Number 273-REG-2015).

Research involving human participants This article contains a study with human participants.

Informed consent The patients were enrolled after having obtained their written informed consent for the use of imaging and the demographic data as educational material and for publications. This study was carried out in accordance with the ethical standards stipulated in the 1964 Declaration of Helsinki and its later amendments and was evaluated by the local IRB.

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