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State-of-the-art technologies provide new insights linking skin and blood vessel abnormalities in SSc-related disorders

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ABSTRACT

Objective: A key unanswered question in systemic sclerosis (SSc) is how microvascular abnormality and fibrosis inter-relate. Our aim was to use state-of-the-art non-invasive imaging methods to gain new insights into pathophysiology, comparing patients with different subtypes of SSc, including early dcSSc, not only to healthy controls but also to patients with causes of Raynaud's phenomenon not progressing to fibrosis.

Methods: Laser Doppler imaging, nailfold capillaroscopy, spectroscopy, and ultrasound measured (respectively) perfusion, microvascular structure, oxygenation/oxidative stress, and skin thickening in the hands of 265 subjects: 31 patients with primary Raynaud's phenomenon (PRP), 35 with undifferentiated connective tissue disease (UCTD), 93 with limited cutaneous SSc (lcSSc), 46 with diffuse cutaneous SSc (dcSSc, including 27 'early') and 60 healthy controls.

Results: Mean perfusion was reduced in SSc groups compared to controls (lcSSc 172 perfusion units [standard deviation 157], late-dcSSc 90 [145], early-dcSSc 68 [137] vs. controls 211 [146]; $p = 0.0002$) as was finger-oxygenation (lcSSc 12.1 [13.6] arbitrary units [AU], late-dcSSc 12.2 [8.4], early-dcSSc 11.1 [11.3] vs controls 14.9 [10.5]; $p = 0.0049$). Oxidative stress was increased at the hand-dorsum in SSc groups ($p = 0.0007$). Perfusion positively correlated with oxygenation ($r = 0.23$, $p < 0.001$), and capillary density negatively with skin thickness ($r = -0.26$, $p < 0.001$).

Conclusion: Our findings lend support to the hypothesis that in SSc, particularly early dcSSc, (but not in PRP or UCTD), reduced perfusion (together with structural microvascular abnormality) associates with reduced oxygenation, with oxidative stress and with skin thickening/fibrosis, most likely driving a vicious cycle which ultimately results in irreversible tissue injury. Findings in skin may mirror alterations in internal organs.

1. Introduction

Systemic sclerosis (SSc) is a multisystem connective tissue disease associated with high morbidity and mortality (Mayes et al., 2003). Much of the morbidity of SSc revolves around the skin involvement, affecting especially hand function; whereas, the mortality is due to internal organ involvement. It is suggested that the characteristic changes occurring in the skin mirror those in internal organs and that fibrosis and microvascular abnormality/dysfunction (leading to reduced perfusion/ischaemia) are key elements of the disease process (Campbell and LeRoy, 1975; Piera-Velazquez and Jimenez, 2015; Lee et al., 2018; Wu et al., 2019; Wannarong and Muangchan, 2018;

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Rodríguez-Reyna et al., 2019; Zanatta et al., 2019). Scleroderma (skin tightening/thickening) is an outwardly visible representation of fibrosis, while severe Raynaud's phenomenon (episodic vasospasm/ischaemia in response to cold or emotional stress) is the most characteristic manifestation of the vascular pathology. Skin fibrosis and cutaneous microvascular structure have been shown to relate to internal organ involvement in several studies, including lung, heart, gut and kidneys (Lee et al., 2018; Wu et al., 2019; Wannarong and Muangchan, 2018; Rodríguez-Reyna et al., 2019; Zanatta et al., 2019); however, at present the inter-relationships between fibrosis and microvascular abnormality are not well understood. It is clear, however, that the microvascular abnormalities are both structural and functional (Campbell and LeRoy, 1975; Herrick, 2000), and that most likely these lead to hypoxia (Beyer et al., 2009; Swigris et al., 2009; Herrick et al., 2010; van Hal et al., 2011) and to oxidative stress, mediated by free radicals (Cotton et al., 1999; Herrick and Matucci, 2001; Ogawa et al., 2006; Kaloudi et al., 2007; Davies et al., 2009; Abdulle et al., 2018). Hypoxia can drive fibrosis by causing upregulation of transforming growth factor-β and endothelin-1 (Hong et al., 2006; Distler et al., 2007; Dooley et al., 2012), thus providing a link between the microvascular and fibrotic elements of SSc.

Novel, non-invasive imaging methods are now available to assess cutaneous oxygenation (including near infra-red and multispectral imaging, (Poxon et al., 2014; Jalil et al., 2018; Gargani et al., 2019; Allen et al., 2018)) and oxidative stress (Allen et al., 2018; Lutgers et al., 2006; Mulder et al., 2006; De Leeuw et al., 2006; Meerwaldt et al., 2007). These allow, for the first time, non-invasive investigation of inter-relationships between these two mechanistic pathways and different SSc-associated cutaneous abnormalities: microvascular structural changes (assessed using nailfold capillaroscopy), reduced perfusion (assessed using laser Doppler imaging) and skin thickening (assessed using high-frequency ultrasound). These imaging methods allow comparisons between patients with SSc, those with primary (idiopathic) Raynaud's phenomenon (PRP, which is an entirely reversible vasospasm), and those with undifferentiated connective tissue disease (UCTD), who are at risk of progressing to SSc but who do not usually have established fibrosis. SSc has two subtypes; limited cutaneous (lcSSc) and diffuse cutaneous (dcSSc, (LeRoy et al., 1988)).

Greater understanding is required of how fibrosis and microvascular structure and function interdigitate to allow elucidation of the underlying pathophysiology and disease process, particularly 1) in the more severe subset of dcSSc, which is often particularly aggressive in the first 5 years (the 'early' phase), with widespread skin thickening and early internal organ involvement and 2) how and why this differs from those with related conditions, such as primary Raynaud's phenomenon and undifferentiated connective tissue disease. Also, there is currently no effective disease modifying therapy for SSc: better imaging techniques may provide objective outcome measures to facilitate clinical trials.

This study aimed to obtain novel insights into the cutaneous aspects of SSc (including oxygenation and oxidative stress) in a large cohort of patients with SSc-spectrum disorders (including early dcSSc, PRP and UCTD). Specifically, we tested the following hypotheses:

a) That patients with SSc, particularly those with early dcSSc, have poorer perfusion, lower density of nailfold capillaries and lower oxygenation and increased oxidative stress and thicker skin compared to healthy controls.
b) That patients with PRP (who do not progress to tissue injury) are, in most respects, similar to healthy controls, and those with UCTD are intermediate between PRP and SSc.
c) For all subject groups together, and in SSc groups only, that relationships exist between measures of blood vessel structure and function and of skin thickening.

Hypotheses a) and b) which considered cross-sectional comparisons between groups (SSc vs controls and PRP and UCTD vs controls), were assessed by comparing measures (perfusion, capillary density, oxygenation, oxidative stress and skin thickness) between groups. Hypothesis c) was assessed by examining (at the finger) inter-relationships between the various non-invasive measures.

2. Patients and methods

Participants attended a single visit in a temperature-controlled laboratory and underwent a 20-min acclimatisation prior to imaging. All were asked to wear light clothing and refrain from vigorous exercise, caffeine and alcohol for 4 h prior to the assessment. The study was approved by NW Research Ethics Committee 6 (11/NW/0244) and all volunteers gave written consent. Patients with PRP were identified as having Raynaud's phenomenon, antinuclear antibody (ANA) titre < 1/100 and normal capillaroscopy. Patients defined as having UCTD had either positive autoantibodies or capillaroscopy changes indicative of an SSc-spectrum disorder, but did not fulfil the criteria for any specific connective tissue disease. Patients with SSc fulfilled either the 2013 American College of Rheumatology/European League Against Rheumatism criteria (van den Hoogen et al., 2013), or the LeRoy and Medsger criteria (LeRoy and Medsger Jr., 2001). Patients with SSc were sub-grouped into those with lcSSc and dcSSc (and separately into early or late diffuse disease: early = within 5 years of first non-Raynaud's clinical feature) (LeRoy and Medsger Jr., 2001). All patients were asked to complete a visual analogue scale (VAS) relating to the level of interference experienced with daily activities due to Raynaud's phenomenon (0 = Raynaud's does not limit activities; 100 = most severe limitation possible). Patients with SSc also completed a digital ulcer VAS, regarding the impact of finger ulcers on daily activities (0 = finger ulcers do not limit activities; 100 = very severe limitation), and an overall impact of disease VAS (0 = no disease impact; 100 = most severe impact imaginable). History of severe finger ischaemia was documented (previous debridement, amputation or intravenous prostanoids). Modified Rodnan skin score (mRSS), a measure of skin thickness (Clements et al., 1995), was assessed by palpation of the non-dominant ring finger and dorsum of the hand (0–3 scale for each of these two sites).

All participants underwent a series of non-invasive measurements/images at the dorsal aspect of the non-dominant finger/hand (Fig. 1). Analysis was carried out by a blinded observer (laser Doppler imaging, high-frequency ultrasound) or via automated software (nailfold capillary measurements, oxygenation and oxidative stress):

2.1. Perfusion

Laser Doppler imaging of the hand was performed (modified-Moor-LDI-vr, 633 nm [3 mW] Moor Instruments, Axminster). Regions of interest were defined in software at the distal phalanx of each finger and dorsum of the hand. The distal-dorsal difference (a perfusion gradient between the finger and the dorsum, Fig. 1b) was calculated as mean finger perfusion minus mean dorsum perfusion (arbitrary perfusion units [PU]) (Anderson et al., 2007; Murray et al., 2004). If fingers are poorly perfused, as typical with Raynaud's phenomenon, there is reduced blood flow; resulting in a negative distal-dorsal difference.

2.2. Microvascular structure

Capillaroscopy (KK systems, Honiton, UK) provided a high-magnification, widefield image of capillaries in the nailbed (Fig. 1a) of the ring finger. Bespoke, automated software identified capillary apices and calculated capillary density (vessels/mm) (Berks et al., 2014; Berks et al., 2018).

2.3. Oxygenation

Oxygenation and oxidative stress were measured using

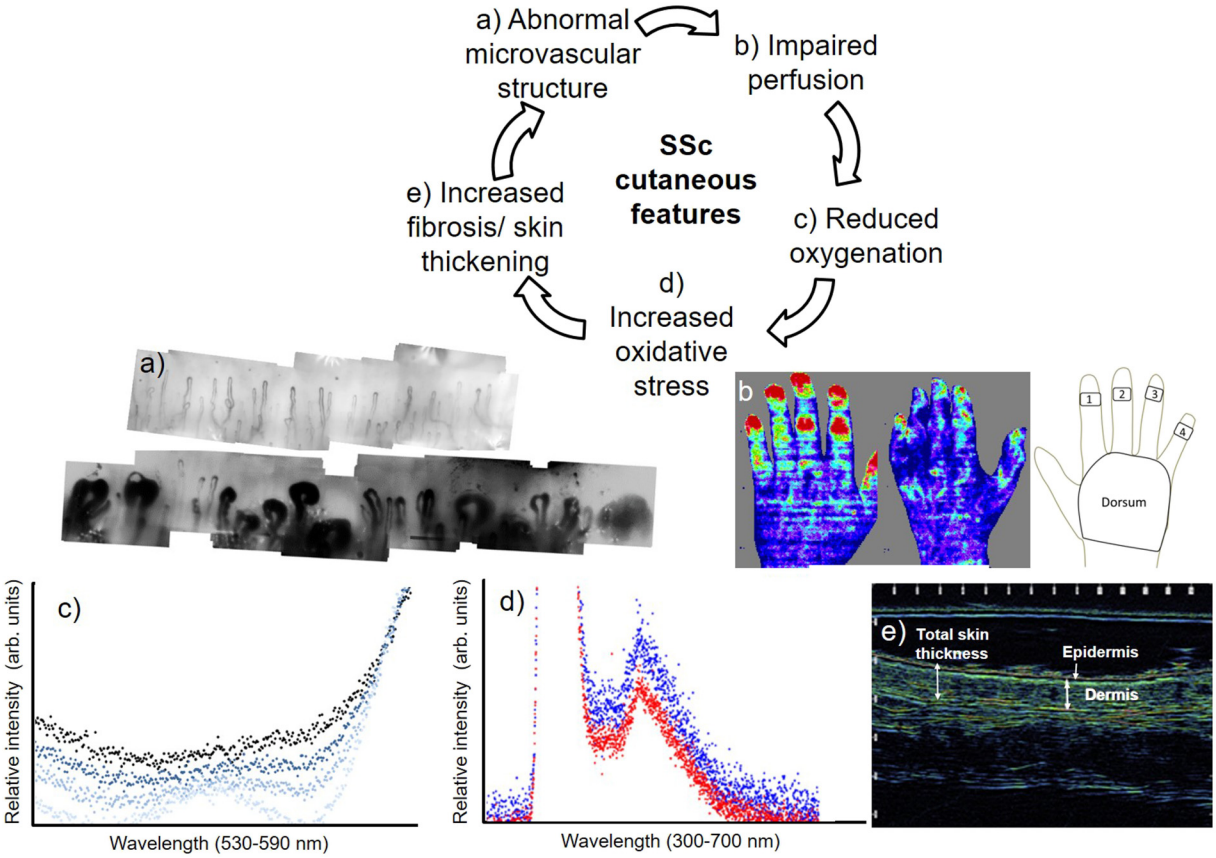

Fig. 1. The hypothesised (vicious) cycle of inter-relationships between different SSc-skin features. The imaging/measurement technique for each feature is shown in the montage below the cycle: a) capillaroscopy showing normal (top) and abnormal capillary structure (below) b) laser Doppler imaging: healthy control (left), patient with SSc (centre), regions of interest for distal-dorsal difference (right) c) white-light absorption spectra of combined oxy- and deoxy-haemoglobin. Occluded finger measurements taken between 30 s (light-blue) to 2 mins (dark-blue [top]); d) UV-spectroscopy. Skin spectra from healthy control (red) and patient with SSc (blue) indicate oxidative stress; e) ultrasound of skin thickness.

spectroscopy. Measurements were taken with a bifurcated fibre-optic probe and spectrometer (USB400 UV–Vis, Ocean Optics, Dunedin, FL, USA). Light was delivered to the skin, and, reflected and back scattered light from the skin was collected by the fibre and delivered to the spectrometer.

For oxygenation measurements the skin was illuminated with white light by the fibre (at a single point, i.e. a local measure). The technique utilises the difference between the light absorption of oxy and deoxygenation haemoglobin within tissue, leading to different light absorption spectra for each and thus a combined curve of haemoglobin that alters dependent upon the relative amount of oxy- and deoxygenated haemoglobin. Oxygenation, in relative arbitrary units, was calculated, using an established technique, from the relative intensity values of 545, 560 and 570 nm, representing points on the curve where absorption is independent of oxygenation and a point of maximum sensitivity to oxygenation. White light spectroscopy measurements were taken at the ring finger (middle phalanx), and centre of hand-dorsum (Fig. 1c). Spectrasuite software was used to capture and save spectra (Ocean Optics). Bespoke software written in Matlab (Mathworks, USA) was used to calculate oxygenation (arbitrary units [AU]) (Murray et al., 2012).

2.4. Oxidative stress

For oxidative stress, measured at the same sites as oxygenation Fig. 1d), an ultraviolet (UV) LED source (372–383 nm, full-width half-maximum) was used with autofluorescence emitted from the skin returning then to the spectrometer. Relative skin autofluorescence was calculated as the ratio of fluorescence emission intensity (430–700 nm) divided by the UV reflection intensity (350–430 nm) to account for any fluctuations in incident UV light levels, as per our previous studies (Murray et al., 2012). The technique is similar to that used in previous studies where measurements of skin autofluorescence, taken at the forearm, have been demonstrated to be a quantitative marker of advanced glycation end products, indicative of over-expressed fluorescent proteins in the skin associated with oxidative stress (Lutgers et al., 2006; Mulder et al., 2006). Data were analysed to give a measure of oxidative stress (arbitrary units [AU]).

2.5. Skin thickness

High-frequency ultrasound images (Longport-Episcan-I-200, 35 MHz, UK) were taken at the same locations as spectroscopy (Fig. 1e). Mean total skin thickness (epidermal plus dermal thickness, [microns]) from three locations per image, was measured using proprietary software.

A set of patient-level measures was derived:

- Laser Doppler: distal-dorsal difference (PU).
- Capillaroscopy: mean capillary density (vessels/mm).
- Spectroscopy (finger and hand-dorsum): oxygenation and oxidative stress (AU).
- Ultrasound (finger and hand-dorsum): total skin thickness (microns).

2.6. Statistical analysis

Hypotheses a) and b). Differences between patient subgroups and controls.

Differences between groups were assessed using one way ANOVA. Where evidence of an overall difference between groups was found, pairwise tests were conducted, subject to Tukey's Honest Significant Difference post-hoc adjustment for multiple testing. *P*-values should be interpreted cautiously in light of the large number of measurements analysed in the study.

Hypothesis c. Relationships between the parameters.

Analysis of the combined group data set: Pearson's correlation was used to compare finger measures to patient VAS and mRSS.

Pearson's correlation between each finger measure and each other finger measure was calculated.

Analysis of the SSc groups: Data for all three SSc groups combined are presented here as scatter plots. Due to small patient numbers no formal statistical analysis was completed.

Data was analysed in STATA 14 (2015 StataCorp LLC, TX, USA).

3. Results

In total, 265 subjects were recruited into the study: 31 with PRP, 35 UCTD, 139 with SSc (93 lcSSc, 19 late-dcSSc, 27 early-dcSSc) and 60 healthy controls (demographics and clinical details in Table 1).

Hypotheses a) and b). Differences between patient subgroups and controls.

Data are shown in Table 2 and Fig. 2.

i) Perfusion (laser Doppler imaging) (Fig. 2a): *a)* Distal-dorsal difference was decreased (i.e. reduced blood flow in the finger compared to dorsum of hand) for all SSc groups; post-hoc analysis indicated statistical significance for patients with early-dcSSc compared with controls. *b)* Post-hoc analysis indicated no statistically significant difference between PRP, UCTD and control groups. PRP and UCTD groups were significantly different to the early-dcSSc group.

ii) Microvascular structure (capillaroscopy) (Fig. 2b): *a)* Capillary density was statistically significantly decreased for all SSc groups as compared to controls. *b)* Post-hoc analysis indicated that PRP, UCTD and controls groups did not statistically significantly differ, but were different to the SSc groups.

iii) Oxygenation (spectroscopy) (Fig. 2c): *a)* Oxygenation at the finger initially appeared decreased in all SSc groups as compared to the control group, but post-hoc analysis indicated no significant difference for SSc versus control. Decreased oxygenation was not observed at the dorsum of the hand for SSc compared to controls. *b)* Post-hoc analysis indicated that, at the finger, oxygenation in patients with PRP and UCTD were not statistically significantly different to controls. Patients with UCTD had higher oxygenation compared to early- and late-dcSSc groups at the finger ($p = 0.0049$).

Table 1
Demographics and clinical features of the different subject groups. Values are number [%] or median [interquartile range, IQR].

		Healthy controls $n = 60$	PRP $N = 31$	UCTD $N = 35$	LcSSc $N = 93$	Late-dcSSc $N = 19$	Early-dcSSc $N = 27$
Demographics	Female, number [%]	42 [70]	25 [81]	27 [77]	79 [85]	14 [74]	18 [67]
	Age, median [IQR] years	48 [40–55]	44 [32–54]	44 [35–52]	61 [55–68]	63 [53–68]	53 [32–58]
	Smoking, number [%]	7 [12]	9 [29]	5 [14]	13 [14]	1 [5]	7 [26]
	Duration of RP, median [IQR] years		7 [3 – 22]	8 [5–15]	18 [11–28]	9 [8–19]	2 [2–5]
	Duration of SSc* median [IQR] years				12 [6–18]	11 [8–19]	2 [1–4]
Current medication, number [%]	Immunosuppressant therapy		0 [0]	3 [9]	9 [10]	5 [26]	8 [30]
	Corticosteroids		0 [0]	2 [6]	13 [14]	6 [32]	10 [37]
	Calcium channel blocker		8 [26]	11 [31]	51 [55]	8 [42]	17 [63]
	Angiotensin-converting-enzyme inhibitor		0 [0]	4 [11]	6 [1]	4 [21]	6 [22]
	Angiotensin II receptor antagonist		1 [3]	3 [8]	11 [12]	5 [26]	1 [4]
	Phosphodiesterase-5 inhibitor		0 [0]	0 [0]	2 [2]	1 [5]	0 [0]
	Endothelin-1 receptor antagonist		0 [0]	0 [0]	2 [2]	3 [16]	0 [0]
	Alpha-adrenergic blocker		0 [0]	0 [0]	3 [3]	0 [0]	0 [0]
Antibodies, number [%]	Anti-Scl-70		0 [0]	1 [3]	7 [8]	6 [32]	8 [30]
	Anticentromere		0 [0]	8 [23]	48 [52]	1 [5]	1 [4]
Clinical characteristics	mRSS fingers (0–3)				1 [1–2]	2 [2–3]	2 [2–3]
	mRSS dorsum of the hand (0–3)				0 [0–1]	1 [1–2]	2 [1–2]
	Severity of Raynaud's VAS (0 – 100)		29 [18–50]	21 [5–50]	22 [6–50]	34 [22–68]	25 [8–71]
	Global disease impact VAS (0–100)				43 [13–68]	51 [25–74]	50 [37–79]
	Impact of ulcers VAS (0–100)				31 [0–80]	42 [0–70]	0 [0–26]
Indicators of severe finger ischaemia, number [%]	History of IV iloprost		0 [0]	0 [0]	22 [24]	10 [53]	8 [30]
	History of finger debridement		0 [0]	0 [0]	20 [22]	5 [26]	1 [4]
	Digital sympathectomy		0 [0]	0 [0]	3 [3]	0 [0]	0 [0]
	Finger ulceration in last year		0 [0]	0 [0]	18 [19]	10 [53]	6 [22]

* defined as duration from first non-RP clinical feature. dcSSc: diffuse cutaneous SSc; lcSSc: limited cutaneous SSc; mRSS: modified Rodnan skin score (Meerwaldt et al., 2007), taken at site of imaging; PRP: Primary Raynaud's phenomenon; SSc: systemic sclerosis; UCTD: undifferentiated connective tissue disease; VAS: visual analogue scale.

Table 2
Non-invasive imaging measures: between group comparisons. Mean (SD) for each measured variable stratified by group, number of data for each imaging technique. P-values from one-way ANOVA are given, following Tukey's Honest Significant Difference post-hoc adjustment. $p < 0.05$ compared to a) control, b) PRP, c)UCTD, d) lcSSc, e) late-dcSSc, f) early-dcSSc respectively. Missing data due to equipment failure or poor quality images/data which did not allow for analysis; for example, movement artefacts on LDI images, non-visible or non-quantifiable vessels in capillaroscopy or low signal to noise in spectroscopy curves. Abbreviations as for Table 1.

Group/parameters Mean (SD)	Healthy controls N = 60	PRP N = 31	UCTD N = 35	lcSSc N = 93	Late-dcSSc N = 19	Early-dcSSc N = 27	p-Value
LDI perfusion parameters							
Distal-dorsal difference (PU)	211 (146)[f] *n = 58*	198 (200)[f] *n = 29*	224 (196)[f] *n = 32*	172 (157) *n = 91*	90 (145) *n = 16*	68 (137)[a,b,c] *n = 24*	0.0002
Nailfold capillaroscopy							
Density (number/mm)	10.8 (2.3)[d,e,f] n = 58	10.2 (2.2)[d,e,f] *n = 31*	9.7 (2.5)[d,e,f] *n = 33*	7.7 (2.6)[a,b,c] *n = 86*	7.3 (3.0)[a,b,c] *n = 15*	7.5 (2.2)[a,b,c] *n = 23*	< 0.0001
Oxygenation (AU)							
Finger	14.9 (10.5) *n = 48*	18.0 (11.9) *n = 24*	22.5 (14.4)[e,f] *n = 31*	12.1 (13.6) *n = 59*	12.2 (8.4)[c] *n = 12*	11.1 (11.3)[c] *n = 11*	0.0049
Dorsum of hand	8.4 (8.9) *n = 49*	10.2 (14.0) *n = 23*	10.1 (15.9) *n = 32*	10.6 (8.8) *n = 61*	9.9 (7.4) *n = 14*	14.5 (6.4) *n = 12*	0.6656
Oxidative stress (AU)							
Finger	4.1 (1.1) *n = 51*	3.6 (0.9) *n = 23*	4.1 (1.6) *n = 28*	4.8 (1.5) *n = 68*	4.7 (1.5) *n = 14*	4.8 (1.4) *n = 14*	0.6103
Dorsum of hand	3.8 (1.0)[d] *n = 53*	3.8 (1.3)[d] *n = 23*	3.7 (0.9)[d] *n = 30*	4.6 (1.2)[a,b,c] *n = 70*	4.2 (1.5) *n = 14*	4.3 (1.4) *n = 14*	0.0007
Ultrasound epidermal and dermal thickness (microns)							
Finger	1173 (267)[d,e,f] *n = 52*	1074 (194)[c,d,e,f] *n = 25*	1312 (234)[b,f] *n = 33*	1390 (217)[a,b,f] *n = 82*	1447 (212)[a,b] *n = 15*	1562 (264)[a,b,c,d] *n = 25*	< 0.0001
Dorsum of hand	1005 (157)[c,d,e,f] *n = 54*	937 (157)[c,d,e,f] *n = 25*	1228 (325)[a,b] *n = 34*	1330 (317)[a,b] *n = 82*	1435 (327)[a,b] *n = 15*	1437 (303)[a,b] *n = 25*	< 0.0001

iv) Oxidative stress (spectroscopy) (Fig. 2d): *a)* There was no difference observed in oxidative stress in the SSc groups as compared to controls at the finger. At the dorsum of the hand, oxidative stress appeared increased for all SSc groups compared to controls; post-hoc analysis indicated statistical significance for lcSSc group versus controls only. *b)* At the finger and dorsum of the hand values for both PRP and UCTD were similar to controls and, at the dorsum, significantly reduced compared to the lcSSc group.
v) Skin thickness (ultrasound) (Fig. 2e): *a)* Skin thickness at the finger and dorsum of the hand were statistically significantly increased in all SSc groups compared to controls; most increased in the early-dcSSc group. *b)* At both the finger and dorsum of the hand, skin thickness was decreased in the PRP group compared to the SSc and UCTD groups. In the UCTD group, finger skin thickness was reduced compared to early-dcSSc, and hand-dorsum thickness increased compared to controls.

Hypothesis c. That in all subject groups, but particularly SSc groups, relationships exist between measures.

3.1. Imaging outcome measures vs patient and clinician reported outcome measures

Pearson's correlation coefficients comparing finger measures in patients with SSc to clinician and patient-reported outcome measures (VAS and mRSS) are shown in Table 3a. Data show no strong correlations. The statistically-significant correlations found were: perfusion negatively correlated to mRSS of the finger ($r = -0.30$; $p < 0.001$) and dorsum of the hand ($r = -0.25$; $p = 0.05$). Thus lower perfusion is associated with increased skin thickening. Capillary density was negatively correlated with global disease impact VAS ($r = -0.21$, $p = 0.02$) and ulcer VAS ($r = -0.22$, $p = 0.01$). Thus those with lower capillary density had worse patient reported outcome measures. Capillary density negatively correlated with mRSS at the finger ($r = -0.23$; $p = 0.01$); i.e. those with lower capillary density had thicker skin. Skin thickness (ultrasound) also correlated with mRSS at both the finger and the dorsum of the hand ($r = 0.29$, $p < 0.05$; $r = 0.26$, $p < 0.05$ respectively).

3.2. Whole data (all groups compared) relationships between imaging outcomes

Correlations between finger imaging outcome measures are shown in Table 3b. R-values, indicative of the 'direction' of a relationship, all support our hypotheses (e.g. perfusion vs. capillary density was positive indicating that in those with increased perfusion, capillary density is higher). Given the large number of non-pre-specified comparisons, we highlight only those with $p < 0.001$.

Distal-dorsal difference positively correlated with finger oxygenation ($r = 0.23$; $p < 0.001$) (i.e. patients with higher finger perfusion had higher oxygenation), and capillary density negatively correlated with skin thickness ($r = -0.26$; $p < 0.001$) (i.e. patients with lower capillary density had increased skin thickness).

3.3. SSc-groups only

Correlations between the finger measures for the combined SSc groups only are shown in Table 3c; Fig. 3 shows example data scatter plots. In the majority of cases the relationships observed in the combined SSc groups as compared to the whole group data followed similar trends, although none reached significance.

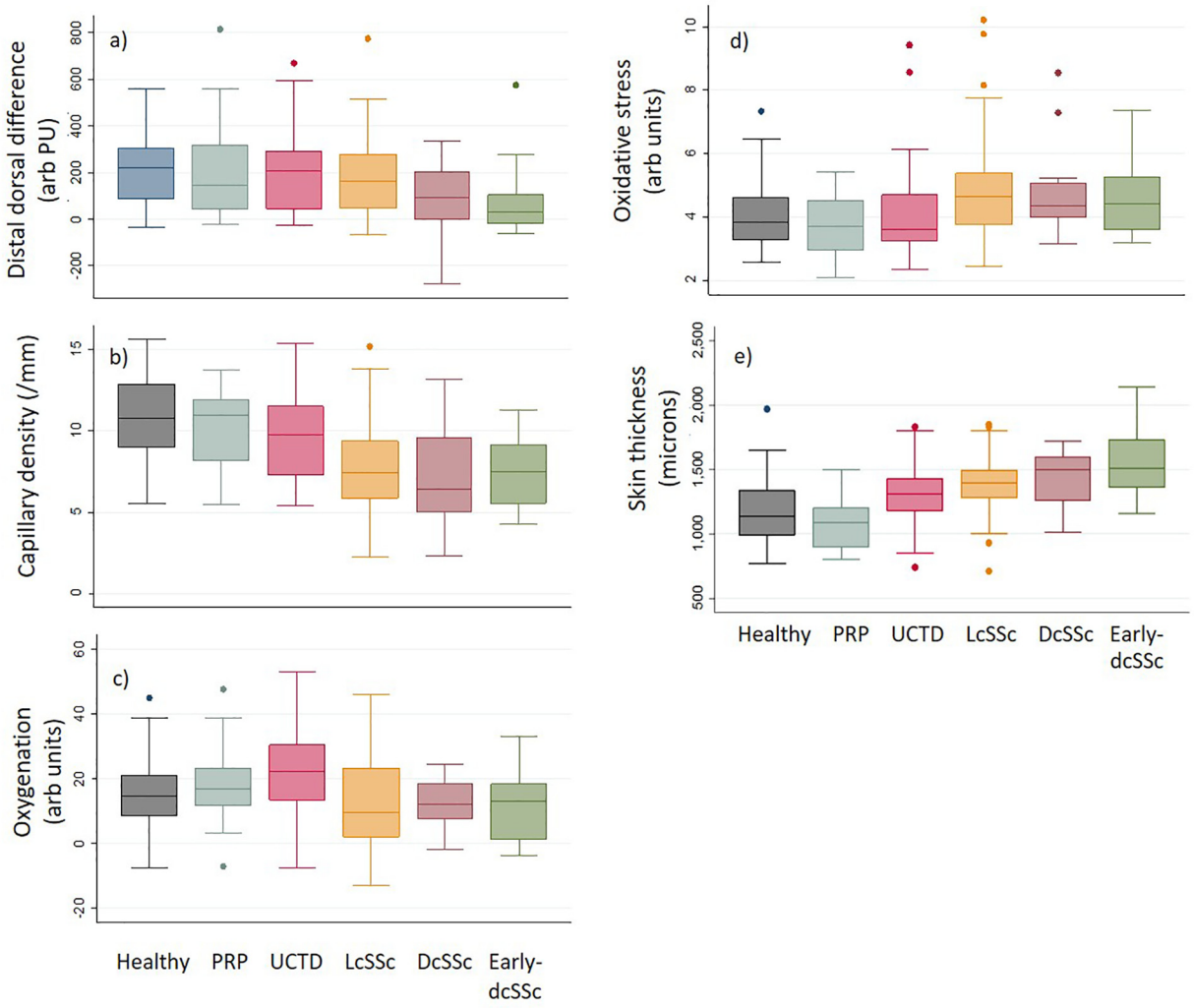

Fig. 2. Box plots of between group comparisons. a) finger perfusion (distal-dorsal difference); b) capillary density; c) finger oxygenation; d) hand-dorsum oxidative stress and e) finger skin thickness. Boxplots show median as central line, IQR as outer box limits, whiskers as maximum and minimum excluding outliers.

4. Discussion

4.1. Patients with dcSSc

Patients with early-dcSSc are of particular interest because patients with this subtype of SSc often have active and progressive disease. It is in this subgroup of patients that there is the greatest need for new treatments (and therefore the greatest need to more fully understand the underlying pathophysiology, in order to identify new treatment targets). The early-dcSSc group showed significantly decreased perfusion compared to the non-SSc groups, (lcSSc and late-dcSSc did not). Capillary density was also significantly decreased compared to non-SSc groups. There was a trend for oxygenation to be decreased and oxidative stress increased compared to non-SSc groups but this was not significant. Rapid, early skin thickening is indicative of poor prognosis (Domsic et al., 2014; Domsic et al., 2011); here skin thickness at the finger was highest in the early-dcSSc group (and statistically significantly increased for all SSc groups compared to controls). Thus it appears, in patients with early-dcSSc, fingers are most affected by low perfusion and increased skin thickness, providing support for the suggestion in the literature (van Hal et al., 2011; Bourji et al., 2015; Gabrielli et al., 2012) (and hypothesised here in Fig. 1) that reduced blood flow may drive fibrosis.

4.2. Patients with PRP and UCTD

Perfusion, capillary density, oxygenation and oxidative stress were not statistically significantly different between the PRP, UCTD and control groups. For the PRP group this was also true for skin thickness. Our results suggest that patients with PRP are similar to controls across all measures. This could help explain why patients with PRP do not progress to irreversible tissue damage: they 'behave' similarly to controls, with no evidence of underlying structural vascular problems leading to reduced oxygenation or oxidative stress. In the UCTD group, skin thickness was greater than in controls, but there was no impairment of oxygenation (which was increased). Longitudinal studies of patients with early UCTD would be of interest, investigating whether low perfusion/oxygenation might be a risk factor for progression to SSc.

4.3. Relationships between skin blood vessel structure and function and skin thickening

Two new key relationships were identified: perfusion has a positive relationship with oxygenation, while capillary density inversely correlates to skin thickness. Other relationships supported our hypotheses but were not highly significant ($p \geq 0.05$). For comparisons within the SSc-only group, the relationships between perfusion, capillary density and skin thickness were maintained but relationships were not significant.

Table 3
Relationships between measurements. Pearson's correlation coefficients (r) and p-values for (a) associations between finger imaging measures in patients with SSc and visual analogue scales (VAS) and modified Rodnan skin score (mRSS) at the finger and dorsum of the hand; (b) associations between non-invasive measures at the finger (including all groups); (c) associations between non-invasive measures at the finger (including all SSc groups).

	Perfusion (distal-dorsal difference [PU])	Capillary density (number/mm)	Finger oxygenation (AU)	Finger oxidative stress (AU)	Finger skin thickness (microns)
	R-value (p-value, N)				
a) Pearson's correlation coefficients for finger measurements with VAS scores and mRSS (all SSc groups)					
Severity of Raynaud's VAS (0–100)	0.02 (0.86)	−0.05 (0.49)	−0.01 (0.91)	−0.00 (0.96)	0.04 (0.58)
	n = 188	*n* = 184	*n* = 133	*n* = 144	*n* = 176
Global disease impact	−0.13 (0.15)	−0.21 (0.02)*	−0.10 (0.39)	0.08 (0.43)	0.08 (0.40)
VAS (0–100)	*n* = 132	*n* = 125	*n* = 83	*n* = 97	*n* = 123
Impact of ulcers	−0.06 (0.51)	−0.22 (0.01)*	−0.04 (0.69)	0.00 (0.97)	−0.05 (0.61)
VAS (0–100)	n = 133	*n* = 126	*n* = 84	n = 98	*n* = 124
mRSS fingers (non-dominant finger	−0.30 (< 0.001)**	−0.23 (0.01)*	−0.15 (0.16)	0.02 (0.84)	0.29 (< 0.05)*
imaged [0–3]) [30]	n = 132	n = 125	*n* = 140	*n* = 98	n = 123
mRSS dorsum of the hand imaged (one	−0.25 (< 0.05)*	−0.10 (0.25)	−0.08 (0.49)	0.06 (0.52)	0.26 (< 0.05)*
hand [0–3])	n = 133	n = 126	*n* = 141	*n* = 99	n = 124
b) Pearson's correlation coefficients for associations between non-invasive measures at the finger (including all groups)					
Perfusion (distal-dorsal difference [PU])		0.05 (0.4)	0.23 (< 0.001)**	−0.00 (0.96)	−0.1 (0.15)
		n = 236	*n* = 175	*n* = 189	*n* = 219
Capillary density (number/mm)			0.09 (0.26)	−0.10 (0.19)	−0.26 (< 0.001) **
			n = 171	n = 184	*n* = 217
Finger oxygenation (AU)				−0.13 (0.08)	−0.05 (0.48)
				n = 173	*n* = 167
Finger oxidative stress (AU)					0.11 (0.14)
					n = 171
c) Pearson's correlation for associations between non-invasive measures at the finger (all SSc groups)					
Perfusion (distal-dorsal difference [PU])		0.01 (0.9)	0.21 (0.06)	0.07 (0.5)	−0.09 (0.37)
		n = 119	*n* = 78	n = 93	*n* = 114
Capillary density (number/mm)			0.03 (0.83)	0.06 (0.57)	−0.16 (0.1)
			n = 72	n = 86	*n* = 109
Finger oxygenation (AU)				−0.09 (0.42)	0.1 (0.36)
				n = 78	*n* = 74
Finger oxidative stress (AU)					−0.01 (0.94)
					n = 81

*p < 0.05, **p < 0.001. In section b) P-values must be considered in the context that there are multiple analyses performed, none of which were pre-specified.

Negative correlation between perfusion and skin thickness has been identified previously in patients with SSc (Ruaro et al., 2018; Sulli et al., 2014), degree of capillary abnormality and skin thickness have been found to positively correlate (Sulli et al., 2014). Other studies have looked for associations between capillary structure (nailfold capillaroscopy) and perfusion (Ruaro et al., 2016; Correa et al., 2010; Camargo et al., 2015; Rosato et al., 2011; Murray et al., 2009). Our data for patients with SSc and healthy controls are consistent with the results of these studies.

Tissue oxygenation in patients with SSc-spectrum disorders has been little studied. Decreased hand-palm oxygenation (assessed using near infrared spectroscopy), was demonstrated in a study of five patients with SSc and five controls (Jalil et al., 2018). In a follow-up study (40 SSc patients and 21 controls) palmar oxygenation did not correlate with capillaroscopy-derived disease severity (Gargani et al., 2019). Transcutaneous oxygenation measures in 12 patients with SSc and 12 controls were similar between groups during cuff-induced ischemia, with diminished oxygenation in patients with SSc (versus controls), during reactive hyperaemia (Broz et al., 2015).

Increased oxidative stress, observed as an increase in skin autofluorescence, has been observed in patients with SSc in previous studies (Allen et al., 2018; Murray et al., 2012; Dadoniene et al., 2015). In previous work we found increased oxidative stress (versus controls) at the proximal finger, hand and forearm but not at the distal finger (Murray et al., 2012). Allen et al. identified increased skin fluorescence and decreased oxygenation in 14 patients with SSc, compared to 9 controls (Allen et al., 2018). Dadoniene et al. measured oxidative stress at the forearm that was associated with changes in finger perfusion under occlusion in 47 patients with SSc versus 47 healthy controls (Dadoniene et al., 2015).

4.4. Strengths and limitations of the study

This study has several strengths. Its large cohort of subjects, including 139 patients with SSc (with a sizeable subgroup of patients with early-dcSSc), as well as patients with PRP and UCTD, has allowed between-group comparisons in unprecedented detail. When studying pathophysiology in patients with (different subtypes of) SSc, we need to compare findings not only to those of healthy controls, but also to those of patients with PRP and UCTD, in order to identify why some but not all patients with Raynaud's phenomenon progress to fibrosis. Another unique strength of the current study was the incorporation of measures of oxygenation and oxidative stress; both more likely to be related to the pathogenesis of fibrosis than measures of blood flow alone. Our method, measuring oxygenation using visible light spectroscopy, has not been applied previously in SSc.

In terms of limitations, we were unable to show, even in our large cohort, that baseline finger oxygenation is definitely decreased in the SSc groups compared to control, PRP and UCTD groups although there was a trend towards reduced oxygenation. In our previous study of oxygenation we have shown that oxygenation measurement is sensitive to changes in digital occlusion in a control group (Poxon et al., 2014). This suggests that the technique is able to measure large differences in oxygenation and shows face validity and sensitivity to change. That we see a small decrease between the SSc and control groups indicates that the technique can detect difference here but that the change is much smaller than during a dynamic change such as an occlusion. Perhaps an occlusion or heating/cooling would have demonstrated a larger difference. Going forwards it may be worthwhile performing a dynamic challenge alongside oxygenation measurement in order to assess differences between groups and relationships between measures.

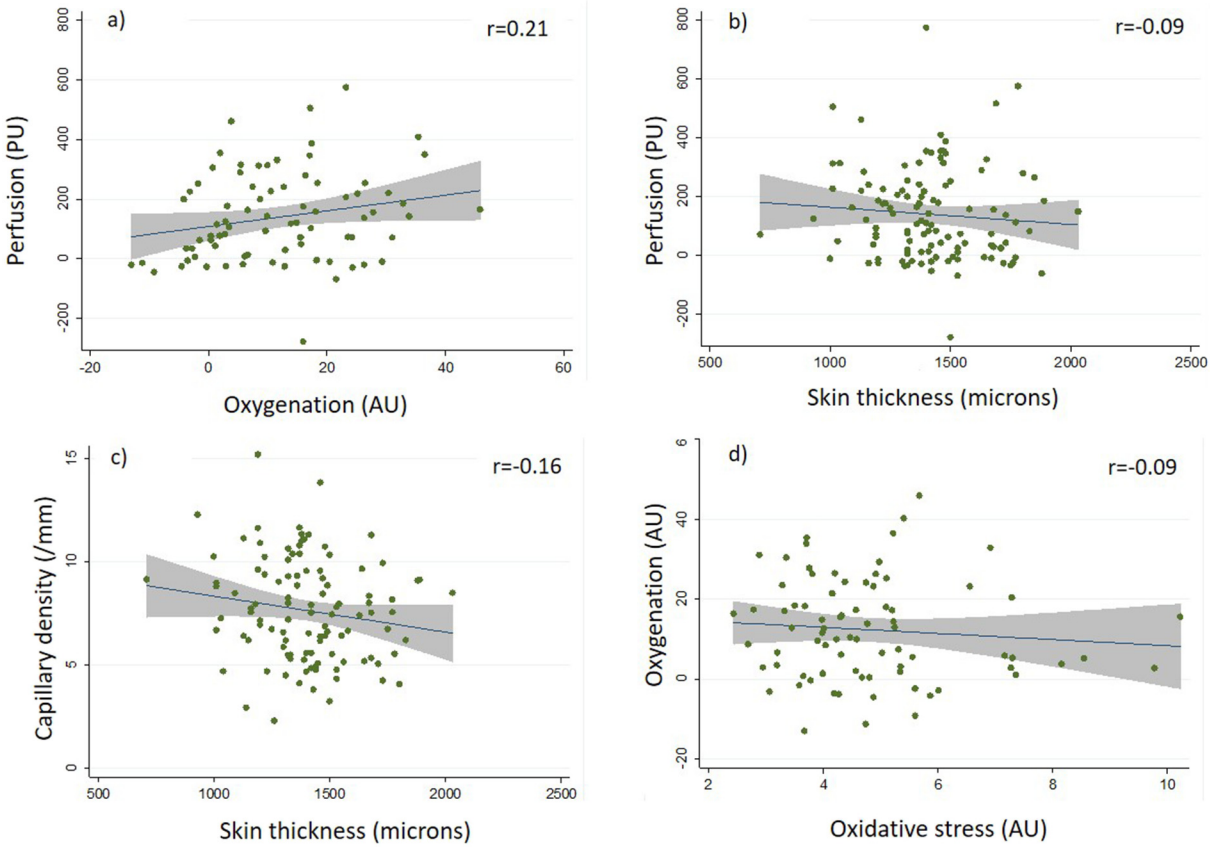

Fig. 3. Examples of scatter plots of the individual associations shown in Table 3. These show the following relationships for the combined SSc groups; a) perfusion and oxygenation; b) perfusion and skin thickness; c) capillary density and skin thickness; d) oxygenation and oxidative stress; the graphs show fitted line of best fit (r-value given) and grey shaded area is the 95% CI.

Similarly, we were unable to show that oxidative stress was increased at the finger in patients with SSc, although there was a significant increase at the dorsum of the hand, in patients in the lcSSc group, and a trend for increase in the dcSSc groups. This finding mirrors our previous finding of increased oxidative stress identified at several skin sites in an SSc vs control cohort but not at the finger (Murray et al., 2012). Our patient group was older than our control, PRP and UCTD groups which potentially may affect our comparisons. However, when these were accounted for as confounders the significance of the data was unaltered (data not shown).

In summary, our findings provide novel insights into the SSc cutaneous disease process, specifically that the structural and functional microvascular abnormalities of SSc are associated with reduced oxygenation and with increased oxidative stress, and that these relate to the extent of skin thickening. Importantly, they demonstrate the feasibility of applying novel non-invasive methods to the study of different aspects of skin pathophysiology. In SSc, the skin reflects the overall disease process. We now have the ability to study inter-relationships between blood flow, oxygenation and fibrosis non-invasively over time, including in response to drug treatment. This is a major step forwards.

Declaration of competing interest

The authors declare no conflicts of interests.

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Author statement

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