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ONLINE SUPPLEMENTARY MATERIAL.

Supplementary material

High frequency ultrasound assessment of systemic sclerosis skin involvement: intra-observer repeatability and relationship with clinician assessment and dermal collagen content

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Supplementary Material 1. METHODS AND MATERIALS

Participant recruitment

Patients fulfilling the 2013 American College of Rheumatology/European league Against Rheumatism classification criteria (1) were enrolled from the SSc clinic at the Royal National Hospital for Rheumatic Diseases, Bath, UK. Exclusion criteria for SSc included the presence of diabetes, current pregnancy or current participation in a clinical drug trial. Previous or concurrent use of DMARDs and vasodilators were permitted. HC (recruited from members of staff and relatives of SSc participants) were additionally excluded if they had a diagnosis of any inflammatory rheumatic disease or Raynaud's phenomenon. All participants underwent clinical and HFUS assessments.

Clinical assessment

All study investigations were performed by the same observer (VF). Participants were asked to refrain from caffeine, smoking and alcohol for at least 4 hours prior to the assessment, which was for the benefit of concurrent vascular assessment that is not reported herein. A clinician case report form collected patient demographics, clinical phenotype and medication use. The local mRSS (2) at each ultrasound region of interest (ROI) and total mRSS was assessed by a single observer (VF). As assessment of atrophic skin is subjective, not captured in the mRSS grading and there is no validated method for reporting such findings, the assessor's opinion on the presence of atrophic skin was not recorded.

Ultrasound Image acquisition

Ultrasound imaging was undertaken on the non-dominant side (so usual daily activities were not affected by wound healing in those undergoing skin biopsy). The image capture protocol was adapted from previous studies (3, 4). All images were obtained

by a single observer (VF) using standardised settings on the same device (Toshiba APLIO A500). All assessments took place before 12pm to minimise the impact of diurnal variation in cutaneous fluid content. Shear-wave elastography (SWE) was assessed using a 14MHz transducer, whereas skin thickness (ST) and echogenicity used a high frequency 18MHz transducer. The transducer was consistently applied perpendicular to the skin surface. The focus was set within the dermis. ROI were assessed at consistent anatomical locations regardless of local mRSS: dorsal aspect of the proximal phalanx of the middle finger, dorsal hand just proximal to the 2nd and 3rd metacarpophalangeal joints, 7-10cm proximal to the wrist over the dorsal forearm, abdominal epigastrium midway between the xiphisternum and umbilicus.

A visible layer of gel was maintained between the transducer and the skin surface for ST and echogenicity to avoid artefactual variable applied pressure. For SWE, the transducer was applied perpendicular to the skin at each ROI using a 2cm solid gel pad standoff (Aquaflex, 04-02, Parker Laboratories Inc., New Jersey, USA) to allow focus within the correct depth of the tissues as well as a thin layer of transmission gel. Adequate but not excessive pressure was applied to maintain contact but avoid external compression of the skin, which would falsely increase elastography readings. Variability of the single observer was assessed for each parameter, at each ROI, in all participants. To do so, the transducer and gel were purposefully removed before repeating the procedure for successive measurements within 5 minutes of the initial assessment

Ultrasound image analysis

Electronic callipers within the APLIO A500 in-built software were manually applied to B mode images to assess ST (millimetres, mm). ST at the hand, forearm and abdomen was measured as the distance between the external surface of the epidermis and dermo-subcutis interface. Whilst the dermo-epidermal junction was visible in most patients it was not always linear. The epidermis was therefore included in ST measurement as judged by the clear external interface with the gel, in line with previous methodologies by others (5-7). Due to challenges identifying the dermo-subcutis junction in some SSc participants, the distance between the external surface of the epidermis down to the finger extensor tendon (clearly visible in all participants) was used to measure skin thickness in the finger, consistent with previous studies (8). Dermal echogenicity was recorded as the mean 'brightness of Gray scale' (scale 0-255) using Image J (https://imagej.nih.gov/ij/), across the whole width of the dermis (to the same depth used for ST measurement), such that low echogenicity suggests tissue oedema and increased echogenicity suggests fibrosis. Images were analysed for ST and echogenicity in batches and without reference to mRSS scores. For SWE, the APLIO A500 in-built software calculated the mean SWE (kPa) within an applied ROI (covering the depth of the dermis and crossing two parallel propagation lines), by means of $E=3pC_s^2$ (E=Young's modulus, p=tissue density, C_s=Shear wave velocity).

Skin biopsy and semi-quantification of collagen density

Optional skin biopsies were obtained from the forearm at the site of HFUS assessment in accordance with a purposive sampling framework that aimed to capture a mix of early/late and limited/diffuse skin changes. Anatomical site and biopsy size (4mm) were chosen to minimize risk to participants in line with EUSTAR guidelines (9) and corresponding with the site of HFUS assessment. Participants on warfarin or direct oral

anticoagulants were excluded from skin biopsy due to the risk of significant bleed in the absence of adrenaline use (a feature included for the benefit of a parallel vascular study). Biopsies were formalin fixed, paraffin embedded (FFPE) and sectioned at 4μm thickness. Skin tissue sections were deparaffinised and stained with Masson's trichrome (Sigma, Saint Louis, USA). Sections were imaged immediately using Leica microscope (CTR40000) and corresponding imaging software (LAS v4.3). Multiple images were taken to cover the whole tissue section (x5 objective) which were 'stitched' together into a single image (CORELdraw v2018, Canada). Collagen staining was quantified using Image J (https://imagej.nih.gov/ij/) to calculate a mean intensity (Gray scale, 0-255) of blue colour across the tissue section, integrated density (mean intensity x blue pixel area) and total sum of the Gray scale (total sum of the intensity of each blue pixel). Histological ST was measured using Image J on the same FFPE sections stained for Masson's Trichrome, with calliper placement at the midpoint of the tissue section. Measurement included epidermis and dermis but not subcutaneous adipose tissue)

RESULTS

Supplementary Table 1. High Frequency Ultrasound assessment of Scleroderma according to clinical grading by local mRSS.

p values illustrated using Kruskal-Wallis. Individual comparison p values between groups (post-hoc Dunn test) are presented in the main manuscript text. Abbreviation: [a]mRSS, modified Rodnan skin score.

HFUS parameter median [IQR]	Healthy control	Systemic sclerosis local mRSS[a]				
		mRSS 0	mRSS 1	mRSS 2	mRSS 3	p value
Proximal Finger, n.	15	11	33	7	2	
Skin thickness (mm)	2.9 [2.6-3.4]	3.0 [2.5-3.8]	3.4 [3.0-4.3]	3.3 [2.1-4.0]	3.6 [3.3 -]	0.137
Echogenicity (Gray scale)	67.0 [55-81]	52.0 [33.0-73.0]	47.0 [40.0-63.5]	50.0 [46.0-60.0]	57.0 [48.0 -]	**0.008**
Elastography (kPa)	33.3 [21.8-40.6]	39.7 [22.5-42.6]	39.6 [32.4-55.5]	49.0 [36.9-73.0]	64.3 [45.6 -]	**0.039**
Distal Hand, n.	15	37	12	3	1	
Skin thickness (mm)	1.4 [1.2-1.5]	1.5 [1.3-1.7]	1.75 [1.2 – 2.0]	2.1 [1.6 – 2.1]	2.1 [-]	**0.037**
Echogenicity (Gray scale)	55.0 [37-74]	46.0 [35.5-61.0]	41.0 [20.3-52.5]	46.0 [35.5 – 57.0]	62.0 [-]	0.234
Elastography (kPa)	27.0 [15.9-40.4]	36.6 [31.3-42.5]	42.5 [31.7-71.8]	45.5 [43.1 – 57.3]	73.7 [-]	**0.007**
Distal Forearm, n.	15	42	9	2	0	
Skin thickness (mm)	1.5 [1.3-1.7]	1.4 [1.2-1.5]	1.6 [1.5-2.1]	1.7 [1.6 – 1.8]	-	**0.012**
Echogenicity (Gray scale)	64.0 [57-71]	74.5 [64.8-87.3]	68.0 [54.5-74.5]	69.5 [59.0 – 80.0]	-	**0.045**
Elastography (kPa)	27.9 [21.1-41.1]	35.1 [27.9-41.0]	39.3 [26.6-46.8]	71.6 [53.8 – 89.3]	-	0.062
Abdomen, n.	15	51	2	0	0	
Skin thickness (mm)	2.0 [1.9-2.5]	1.8 [1.5-2.0]	2.4 [2.2 – 2.6]	-	-	**0.008**
Echogenicity (Gray scale)	62.0 [49-81]	69.0 [62.0-86.0]	62.5 [41.0 – 84.0]	-	-	0.208
Elastography (kPa)	15.8 [9.4-25.9]	25.9 [19.6-29.2]	51.5 [17.1-85.8]	-	-	**0.035**

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