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Novel optical assessments of tissue composition and viability using fluorescence spectroscopy and tissue oxygenation spectrophotometry in patients with systemic sclerosis - a pilot study

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Novel optical assessments of tissue composition and viability using fluorescence spectroscopy and tissue oxygenation spectrophotometry in patients with systemic sclerosis - a pilot study

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Lesley Ottewell³ and Bridget Griffiths³

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Abstract

Patients with systemic sclerosis (SSc) experience significant morbidity and mortality, therefore, the development of tests to aid its early diagnosis are very important. The aim of this pilot study was to assess the diagnostic value of novel optical non-invasive skin fluorescence spectroscopy (FS) and tissue oxygen saturation (TOS) viability measurements in patients with established SSc. Two groups were studied, comprising 14 SSc patients and 9 healthy controls (93% and 73% females, respectively). FS and TOS measurements were collected from 3 body sites: the forearm, chest, and calf. Fluorescence intensities at wavelengths attributed to collagen, elastin, and L-tryptophan were computed, with adjustment for melanin, and a normalised combined fluorescence score (NCFS) was determined. The NCFS was significantly higher ($p < 0.001$) and the combined TOS significantly lower ($p < 0.001$) in the SSc group. TOS measurements alone showed good classification accuracy (95.7%) at separating SSc from healthy control participants, with some clustering of values close to the 50% oxygenation level in both groups. When the composition and viability measures were combined and modelled using binary logistic regression, excellent results for the sample were obtained following leave one out cross validation (100%). The results of this pilot study demonstrate the potential diagnostic utility of FS and TOS assessments in SSc patients and further work is now needed to validate these techniques prospectively in a larger group of SSc patients across the spectrum of the disease, and also patients with other types of vasculopathy and conditions that can cause skin fibrosis.

Keywords: Systemic sclerosis, Biophotonics, Fluorescence spectroscopy, Tissue oxygen saturation, Connective Tissue Disease

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Introduction

Systemic sclerosis (SSc), also known as scleroderma, is a connective tissue disease with heterogeneous multi-system involvement, characterised by the presence of fibrosis, auto-antibodies, and vasculopathy [1, 2]. Fibrosis is a hallmark of SSc and it leads to the replacement of the normal tissue architecture with stiff, collagen-rich connective tissue, resulting in skin thickening [3]. An early diagnosis would help implement the most appropriate treatment for each patient, i.e. the early initiation of immunosuppressants and/or vasodilator drugs, as clinically indicated, thus improving quality of life and survival [4]. At the moment, however, there is a paucity of sensitive clinical tools for the early assessment of scleroderma.

Nailfold capillaroscopy (NFC) is usually performed to investigate the presence of microangiopathy secondary to connective tissue disease, particularly in SSc [5]. This methodology, however, does not traditionally give a quantitative measure of abnormality for clinical use, despite some recent developments in this area [6, 7], and typically relies on the skills and experience of clinical specialists in the technique. Another technique that has shown promise is optical coherence tomography (OCT), which can provide a virtual biopsy of the superficial layers of the skin [8, 9]. Other potentially useful and non-invasive alternatives to NFC and OCT are optical technologies such as fluorescence spectroscopy (FS) and tissue oxygen saturation (TOS).

FS provides an “optical biopsy” of the skin [10, 11] and has been preliminary assessed in patients with diabetes [12-14] and systemic sclerosis [15, 16]. It can indirectly assess the amount of connective tissue (including collagen and elastin) in the measurement volume, as the higher the intensity, the higher the amount of fluorophore in the sample volume [17]. It is also sensitive to other key biomarkers such as L-tryptophan, and the relative fluorescence intensities between the various components can provide additional information. This technology would allow for the objective assessment of the changes in the composition of the connective tissue typical of SSc. TOS provides an objective assessment of tissue perfusion [18] and with microvascular perfusion characteristically impaired in patients with SSc [19],

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3 this would provide valuable information regarding the viability of the superficial layers of
4 tissue.
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8 The aim of this pilot study was to assess the clinical utility of novel optical non-invasive skin
9 FS and TOS measurements to discriminate between healthy controls and patients with
10 established SSc.
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Methods

Participants

Two groups were studied in this pilot, comprising patients with SSc and healthy control (HC) participants. The SSc group fulfilled the American College of Rheumatology (ACR) preliminary classification criteria for systemic sclerosis [20]. The HC control group had no known major cardiovascular disease (hypertension, ischaemic heart disease, stroke, or peripheral vascular disease), no diabetes mellitus, no Raynaud's phenomenon, and were not taking Vitamin D medication. All participants across both groups were of white Caucasian ethnic origin. The study was approved by a National Health Service (NHS) Research Ethics Committee (REC) (County Durham and Tees Valley 2, 07/Q1001/46), and all participants gave their written informed consent prior to participation in the study.

Systolic and diastolic blood pressures were measured manually using a sphygmomanometer device (Accoson Greenlight™ 300, Harlow, UK). Height, weight, and cardiovascular history were also recorded for all of the participants. In the SSc group, their SSc clinical history, including disease duration (defined as the onset of the first non-Raynaud's manifestation), sub-group (limited cutaneous [lcSSc] or diffuse cutaneous [dcSSc] disease), and modified Rodnan skin score (mRSS) were also documented [21, 22].

Vascular optical measurements

The Freeman Hospital microvascular measurement protocol for subject preparation was followed [23]. This included no caffeine, nicotine, exercise, hot drinks, or substantial food in the four hours before measurements, as well as no cosmetic skin cream or talc to the hands, arms, or legs on the day of the test. Participants were given the opportunity to acclimatise in the normothermic (temperature $23.5\pm 0.5^{\circ}\text{C}$, relative humidity 40-50%) temperature-controlled measurement room for a period of at least 15 minutes prior to their measurements.

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3 FS and TOS measurements were made by a single operator (JA) at three body sites: the
4 forearm (over the dorsal aspect, 3 cm proximal of the wrist on the non-dominant arm); the
5 chest; and the calf (over the lateral aspect of leg, 12 cm proximal of the ankle joint on the
6 non-dominant leg). Measurements were made in minimal ambient lighting conditions to
7 reduce the risk of interference.
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10 11 12 13 *Tissue fluorescence for tissue composition* 14

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17 A FS system (SPEX-2 Fluoromax, Horiba Jobin-Yvon, Stanmore, UK) was used to measure
18 the skin tissue fluorescence excitation-emission characteristics. The study wavelengths
19 comprised an excitation range of 260-500 nm and corresponding emission range of 290-750
20 nm. This range includes the three key regions relative to collagen (C) type I (located at
21 wavelengths of 340 nm excitation and 390 nm emission), elastin (E) (370 nm excitation, 440
22 nm emission), and L-tryptophan (T) (290 nm excitation, 350 nm emission) [24, 25]. The
23 excitation and emission spectra were processed to form an excitation-emission matrix (EEM)
24 [11], from which specific fluorophore intensities were extracted using MATLAB software
25 developed in-house by JA (The Mathworks Inc., Natick, MA, USA). Fluorescence intensities
26 were measured as counts of photons per second (cps). Figure 1 shows an example EEM with
27 the three fluorophore wavelength areas indicated.
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38 Fluorescence assessments are biased by skin colour, and in order to compensate for this, a
39 colorimeter (Konica Minolta type CM-508i, Warrington, UK) was used to objectively
40 quantify skin colour from reflectance data. The key compound affecting skin colour is
41 melanin which exhibits a reflectance peak at 660 nm [26]. This information was extracted to
42 form a Melanin Index (MI) which corrected the values returned by FS. The Melanin Index
43 was computed as described in equation 1 [27].
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$$50 \quad \text{MI (in \%)} = 100 \times \log_{10} (100 / \text{reflectance at 660 nm}) \quad (1)$$

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53 Fluorescence cps were multiplied by MI/100 to give melanin normalised T_{MI} , C_{MI} and E_{MI}
54 values at each body site. These values were then combined across the three body sites for
55 each of the three fluorophores as a summation, with the example of collagen described in
56 equation 2.
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$$C_{MI \text{ combined}} = C_{MI \text{ forearm}} + C_{MI \text{ chest}} + C_{MI \text{ calf}} \quad (2)$$

A normalised combined fluorescence score (NCFS) for connective tissue was then calculated as described in equation 3 to give a dimensionless index for each participant.

$$NCFS = (C_{MI \text{ combined}} + E_{MI \text{ combined}}) / T_{MI \text{ combined}} \quad (3)$$

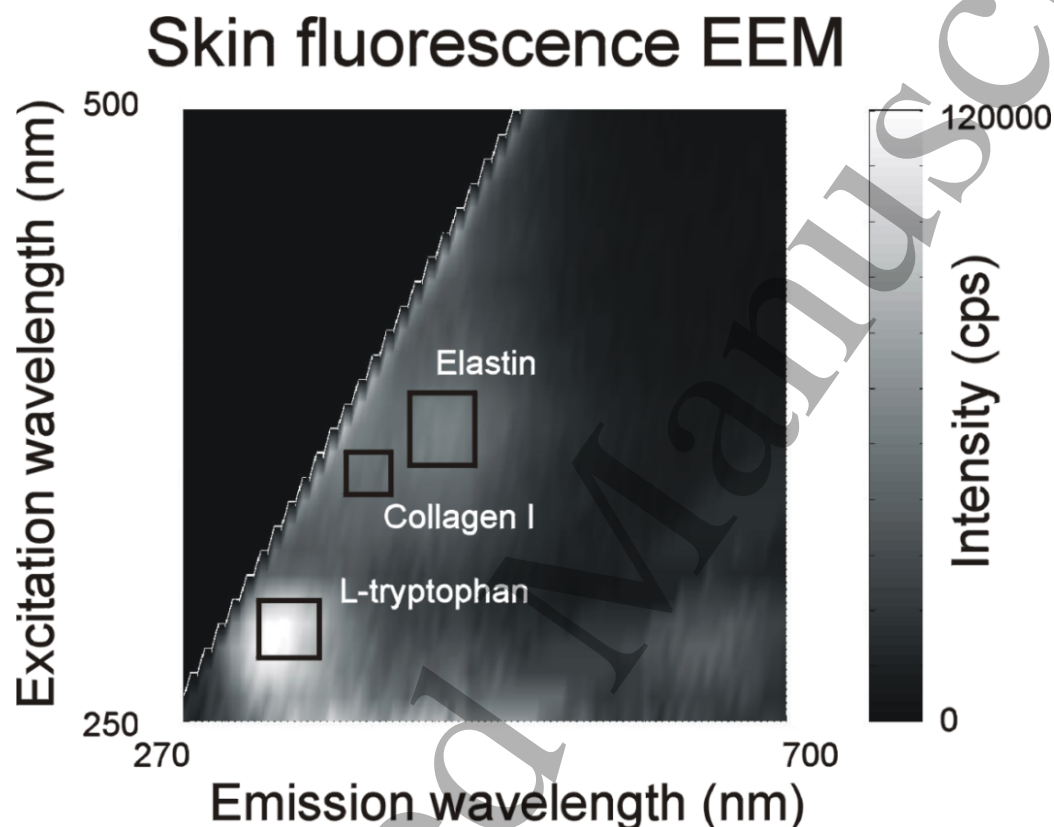


Figure 1 - Example fluorescence spectroscopy (FS) excitation-emission matrix (EEM) for a single body skin measurement site. The black squares indicate the foci from where the three key wavelength regions from which L-tryptophan, collagen (type I), and elastin fluorophore intensities were extracted. The fluorescence intensity scale in counts per second (cps) is added vertically to the right hand side of the figure.

Tissue oxygen saturation for tissue viability

TOS [28, 29] was measured using an O2C system (LEA Medizintechnik GmbH type O2C, Giessen, Germany) using a LMF-2 flat skin probe. Although the specific algorithm used by the device is proprietary to LEA, the technique is based on deriving the superficial TOS from the profile of the reflection spectra across a range of visible wavelengths close to green. The

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TOS values from each participant were combined across their three body sites using a simple mean representation.

Statistical analysis

Demographic, clinical, and optical measurements were summarised for each participant group using non-parametric descriptive statistics, i.e. median [inter-quartile range] values. The differences between participant groups in numerical measures were assessed for statistical significance using the Mann-Whitney U-test, and binary categorical counts using the Fisher's exact test. A significance level of 95% was considered statistically significant. A classification model (SSc vs HC) was produced using binary logistic regression (BLR), with NCFS and the mean TOS used as predictors [30]. To investigate the model stability and generalisability outwith the current sample, leave one out cross validation (LOOCV) was used. The LOOCV adjusted predicted probabilities for a participant having SSc, returned from the BLR models, were used to develop a novel diagnostic disease probability plot, with linear interpolation used to smooth boundaries. Receiver operating characteristic (ROC) analysis was performed to assess the diagnostic performance of the model using the LOOCV adjusted predicted probabilities. All statistical analyses and plots were produced using the statistical programming language R (version 3.1.1), via the integrated development environment RStudio (version 0.99.446).

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Results

Demographic and clinical characteristics

A summary of the demographics and clinical characteristics for the 14 SSc and 9 HC participants are presented in Table 1. There were no significant differences between the two groups across all demographics and clinical characteristics, although significance was almost reached in age (61 years [54-64] versus 52 [48-55]) and diastolic blood pressure (70 mmHg [61-86] versus 78 [72-78]).

Table 1 – Summary of demographics and clinical characteristic of the systemic sclerosis (SSc) and healthy control (HC) groups.

	SSc (n = 14)			HC (n = 9)			p value
	Median (n)	[IQR] (%)	or	Median (n)	[IQR] (%)	or	
Female (n)	(13)	(93)		(7)	(78)		0.53
Age (years)	61	[54-64]		52	[48-55]		0.07
SBP (mmHg)	112	[106-125]		122	[112-124]		0.47
DBP (mmHg)	70	[61-75]		78	[72-78]		0.05
Height (m)	1.64	[1.61-1.68]		1.67	[1.66-1.73]		0.16
Weight (kg)	70	[61-86]		66	[66-82]		0.95
BMI (kg/m ²)	26.1	[22.9-30.5]		24.0	[23.1-26.2]		0.47
Current smokers (n)	(0)	(0)		(1)	(11)		No comp
Diabetes (n)	(2)	(17)		(0)	(0)		No comp

'SBP' = 'Systolic blood pressure'; 'DBP' = 'Diastolic blood pressure'; 'No comp' = 'No comparison from Fisher's exact test possible'

In the SSc group, patients had a median [IQR] mRSS of 7 [4-15], an SSc duration (in years) of 6 [3-8], with 7 patients (50%) having limited SSc, and 7 patients (50%) having diffuse SSc. 11 patients (79%) also had positive immunology identified by anti-centromere antibody (4 patients), anti-topoisomerase I (2 patients), RNA polymerase III (1 patient), and anti-nuclear antibody (4 patients). Six patients with SSc had interstitial lung disease (ILD).

Tissue fluorescence - composition

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Melanin

In the SSc group, the median [IQR] melanin index at the forearm was 28.9% [26.4-31.2], chest 32.8% [29.1-39.3], and calf 26.0% [23.5-26.8]. In the HC group, the median [IQR] melanin index at the forearm was 28.4% (26.9-31.0), chest 27.2% (26.1-32.2), and calf 25.2% (24.4-27.2). The melanin indices were used to adjust the L-tryptophan, collagen, and elastin fluorophores for tissue pigmentation.

Fluorophores – melanin normalised

Table 2 presents a comparison of the melanin normalised tissue fluorescence values for T_{MI} , C_{MI} , and E_{MI} across the body sites and between the SSc and HC groups.

Table 2 – Comparison of the melanin normalised tissue fluorescence values (k counts of photons per second (cps)) for L-tryptophan (T_{MI}), collagen (C_{MI}), and elastin (E_{MI}), across the body sites of the forearm, chest, calf, combined sites, and the normalised combined fluorescence score (NCFS) between the systemic sclerosis (SSc) and healthy control (HC) groups.

	SSc (n = 14)		HC (n = 9)		p value
	Median	[IQR]	Median	[IQR]	
L-tryptophan (T_{MI})					
-Forearm	96	[59-133]	71	[52-92]	0.602
-Chest	17	[11-25]	9	[7-31]	0.688
-Calf	163	[130-216]	307	[271-319]	0.004*
-Combined (sum)	269	[234-370]	373	[354-458]	0.062
Collagen (C_{MI})					
-Forearm	146	[122-175]	95.7	[86-111]	0.062
-Chest	171	[107-248]	74.3	[50-126]	0.045*
-Calf	283	[188-336]	296	[248-440]	0.403
-Combined (sum)	610	[464-738]	473	[420-682]	0.369
Elastin (E_{MI})					
-Forearm	327	[296-407]	237	[198-255]	0.016*
-Chest	388	[278-610]	157	[104-260]	0.009*
-Calf	530	[402-652]	474	[360-646]	0.734
-Combined (sum)	1302	[1061-1530]	851	[771-1126]	0.023
NCFS	6.49	[5.53-9.76]	3.53	[2.92-4.52]	< 0.001*

* = significant at the 0.05 level

When the body sites were combined, there were no significant differences in T_{MI} and C_{MI} ($p = 0.062$ and $p = 0.369$, respectively) between SSc patients and HCs. There was a significant difference in E_{MI} between groups ($p = 0.023$), with SSc patients having greater E_{MI} levels

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3 than healthy controls (1302 [1061-1530] versus 851 [771-1126]). There was also a significant
4 difference in NCFS between the groups, with SSc patients having a greater NCFS than HCs
5 (6.49 [5.53-9.76] versus 3.53 [2.92-4.52]).
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10 Tissue oxygen saturation - viability

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14 Table 3 presents a comparison of TOS across the body sites and between the SSc and HC
15 groups. A combined tissue oxygen value for the 3 sites is also quantified and compared.
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20 There were significant differences between participant groups in the TOS at the forearm,
21 chest, and calf, and then when combined ($p < 0.001$, $p = 0.029$, $p < 0.001$, $p < 0.001$, respectively),
22 with SSc patients having significantly reduced TOS than HCs (Table 2).
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27 **Table 3** – Comparison of tissue oxygen saturation (TOS) across the body sites of the forearm,
28 chest, calf, and combined sites between the systemic sclerosis (SSc) and healthy control (HC)
29 groups.
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	SSc (n = 14)		HC (n = 9)		p value
	Median	[IQR]	Median	[IQR]	
-Forearm (%)	37	[32-46]	58	[57-61]	< 0.001*
-Chest (%)	56	[49-61]	70	[56-73]	0.029
-Calf (%)	26	[21-31]	49	[44-66]	< 0.001*
-Combined (%) (mean TOS)	42	[36-45]	56	[54-66]	< 0.001*

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40 * = significant at the 0.05 level
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45 Combined fluorescence spectroscopy and tissue oxygen saturation assessments

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48 Figure 2 depicts a disease probability plot displaying the LOOCV adjusted predicted
49 probability for a participant having SSc returned from a BLR model (Group ~ NCFS + Mean
50 TOS).
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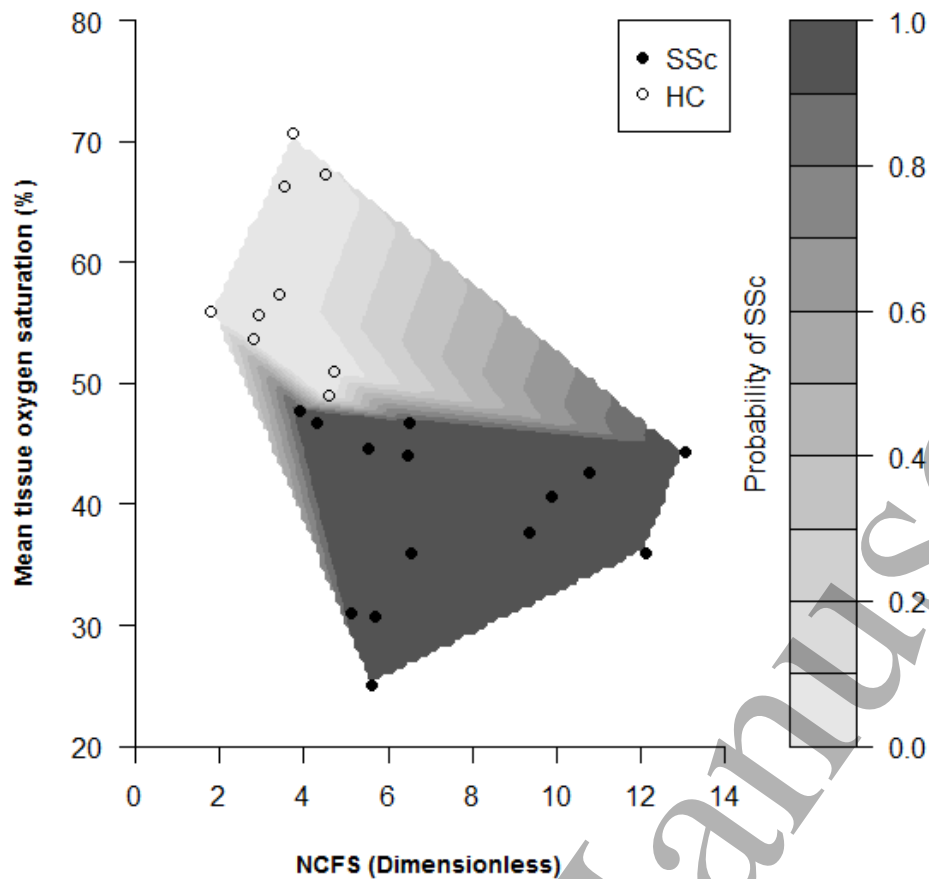


Figure 2 – Novel diagnostic disease probability plot displaying the leave one out cross validation (LOOCV) adjusted predicted probability for a participant having systemic sclerosis (SSc) returned from a binary logistic regression (BLR) model ($SSc/HC \sim NCFS + \text{Mean tissue oxygen saturation (TOS)}$).

Figure 3 presents the diagnostic performance of the BLR model using LOOCV adjusted predicted probabilities for a participant having SSc. The optimal LOOCV adjusted predicted probability threshold was 0.956 (Figure 3). At this threshold, the accuracy, sensitivity, specificity, negative predictive value (NPV), and positive predictive value (PPV) of the model were all 100%.

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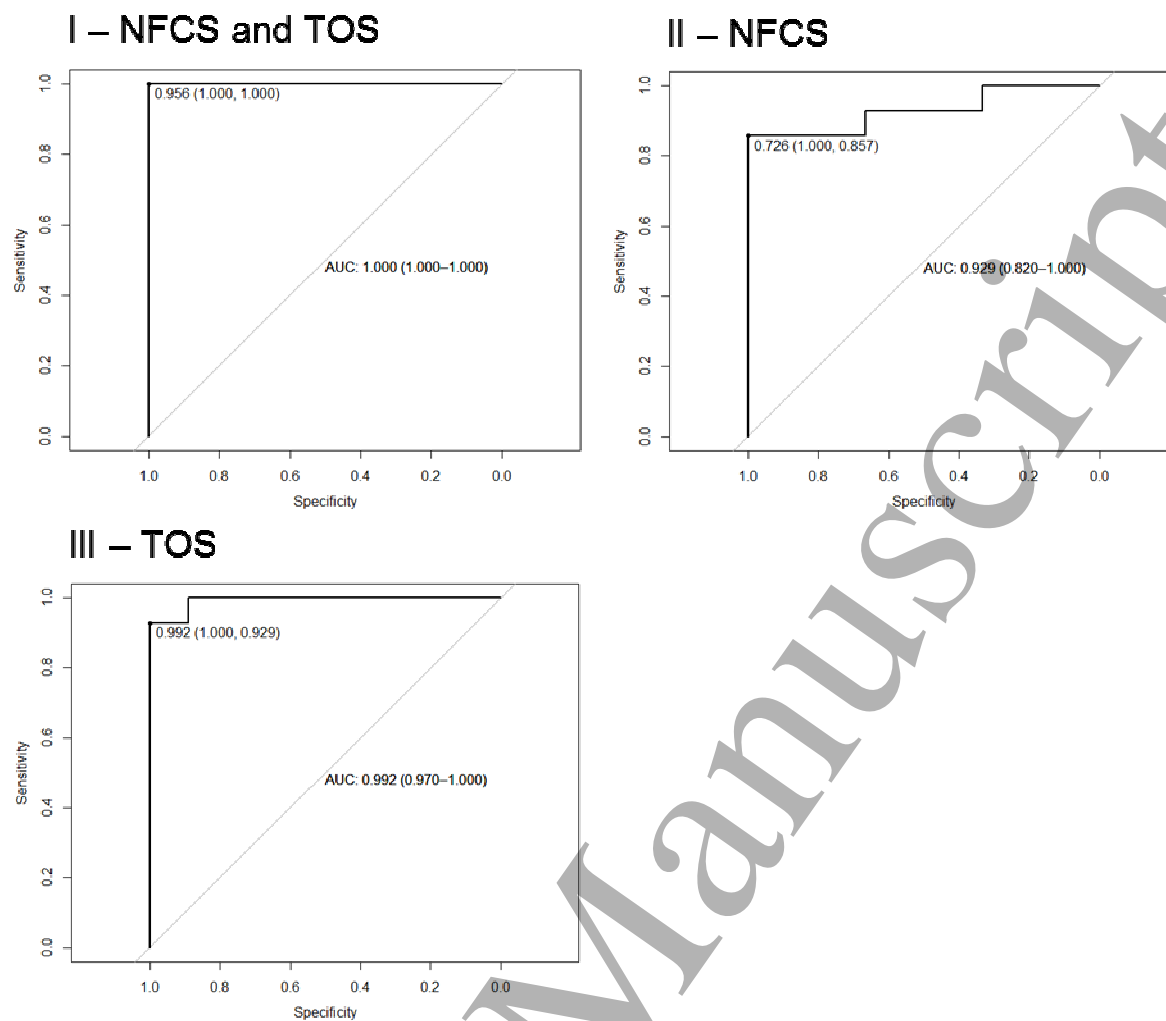


Figure 3 – Receiver operating characteristic (ROC) curves displaying the diagnostic performance of the binary logistic regression (BLR) models, using leave one out cross validation (LOOCV) adjusted predicted probabilities, for a participant having systemic sclerosis (SSc) using the following predictors: I – normalised combined fluorescence score (NCFS) and mean tissue oxygen saturation (TOS); II – NCFS; III – TOS. The optimal LOOCV adjusted predicted probability threshold (specificity, sensitivity) and area under the curve (AUC) values (95% confidence interval) are shown.

For NCFS used alone, the optimal LOOCV adjusted predicted probability threshold was 0.726. At this threshold, the accuracy of the model was 91.3%, sensitivity 85.7%, specificity 100%, PPV 100%, and NPV 81.8%. For TOS used alone, the optimal LOOCV adjusted predicted probability threshold was 0.992. At this threshold, the accuracy of the model was 95.7%, sensitivity 92.9.7%, specificity 100%, PPV 100%, and NPV 99.2%.

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Discussion and Summary

Our pilot study, using a non-invasive combined fluorescence score (NCFS) and TOS measurements over 3 different skin sites on the body, has shown that in SSc there is significantly increased fluorescence associated with connective tissue whilst tissue oxygenation levels are significantly reduced. TOS measurements alone showed good classification accuracy, after cross validation (95.7%), for separating SSc patients from HCs, although there was some clustering of values close to the 50% oxygenation level in both groups. When the composition and viability measures were combined and compared using a BLR classification model, excellent classification accuracy after cross validation was observed (100%). The results of this pilot study demonstrate the potential diagnostic utility of FS and TOS assessments in SSc patients, and further work is needed to validate these techniques prospectively in a larger group of SSc patients and also patients with other conditions that can cause skin fibrosis.

Another previous study that assessed tissue FS in patients with systemic sclerosis reported significant differences in fluorescence at some sites along the arm, although with overlap in measurements between the participant groups [16]. In this study, we have made advances to the method in various ways: developing the combined fluorescence score (NCFS) which represents measurements from 3 skin sites across the body to give a wider picture of tissue composition changes, including an adjustment for tissue melanin since skin pigmentation is known to change in patients with SSc; exploring the value of combining FS with TOS measurements to enhance the diagnostic accuracy; and introducing a novel disease probability plot to aid the visual interpretation of the two measurements.

The results of increased connective tissue in SSc patients, i.e. collagen and elastin, are consistent with the well-known presence of fibrosis in these patients; as microvascular oxygenation is characteristically impaired in patients with SSc. Resulting hypoxia can lead to an increase in collagen production by the fibroblasts with altered transforming growth factor beta (TGF β) function a key factor in the increased fibrosis. TGF β is pleiotropic and as well as stimulating fibroblasts to stimulate collagen synthesis, it also stimulates the synthesis of elastin [31, 32]. We have looked at two pathways both of which are important in the pathogenesis of SSc, i.e. vascular and fibrosis. A third possible pathway to explore would be

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3 immunological markers, with our method having the potential to improve classification in
4 those who have SSc but with non-specific or negative immunology. In this study, only half of
5 the SSc patients had specific immunological markers associated with the condition, although
6 nonspecific antibodies were present in other patients.
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11 Interestingly, neither fluorescence nor TOS correlated significantly with the validated mRSS
12 which evaluates skin thickness, which will need to be explored further. The method will also
13 need to be assessed in a larger cohort of patients with SSc, including patients with newly
14 diagnosed disease, although some patients with early SSc were included in this study. The
15 novel diagnostic disease probability plot (Figure 2) also needs to be tested prospectively, and
16 if the same level of diagnostic performance were to be maintained in patients with early
17 phase SSc, this would be very beneficial and facilitate early treatment with
18 immunosuppressants and/or vasodilator drugs as clinically indicated. However, this needs to
19 be tested in a wider study of early, active, and late phase SSc patients. Other patient groups
20 should also be tested, including patients with primary Raynaud's phenomenon and patients
21 with pre-scleroderma, i.e. patients that have Raynaud's and the specific antibodies seen in
22 SSc, but who have no other clinical features of SSc. Following this group longitudinally and
23 seeing if these measurements could predict who will develop SSc would also be interesting
24 and of potential clinical importance. In addition, a further study should include a wider cohort
25 to allow the assessment of non-Caucasian participants, a range of vascular pathologies
26 including arteriosclerosis and a range of patients with diabetes, as well as participants with
27 calcinosis and interstitial lung disease (ILD) using the two optical techniques [33]. Additional
28 clinical information, such as the hormonal activity of female participants, should also be
29 captured due to the potential effect of the menstrual cycle on results. Other techniques, such
30 as OCT [8, 9] should also be considered to help investigate the utility of biophotonic
31 techniques for SSc assessments.
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50 FS and TOS measurements are efficient, requiring only a few minutes per body site and could
51 be made faster and lower cost with the latest technology options in FS and TOS using
52 spectrophotometry. The technologies are safe and pose no risk to the patient when the correct
53 optical procedures are followed. The techniques offer potential value in SSc diagnostics,
54 screening, monitoring response to treatment, and may complement existing validated
55 methods such as nailfold capillaroscopy. In summary, in this pilot study, the normative
56 ranges for the combined fluorescence score (NCFS) and TOS have been estimated for the
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three body measurement sites and provided high diagnostic classification accuracies for SSc patients. A further larger study is required to validate these findings.

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