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Limited Cutaneous and Diffuse Cutaneous Scleroderma: Circulating Biomarkers Differentiate Lung Involvement

Kevin J Keen[1]*, Stephan F van Eeden[2] and James V Dunne[3]

[1]Department of Mathematics and Statistics, University of Northern British Columbia, Prince George, Canada
[2]Division of Respiratory Medicine, Department of Medicine, University of British Columbia, Vancouver, Canada
[3]Division of Rheumatology, Department of Medicine, University of British Columbia, Vancouver, Canada

Abstract

Study background: Insufficient or absent angiogenesis are hallmarks of scleroderma (SSc). Microvascular change is an early manifestation of SSc followed by intimal proliferation and fibrosis of arterioles resulting in reduced blood flow and tissue ischemia. This ongoing vasculopathy in the lungs presents clinically as pulmonary hypertension and characteristically precedes lung fibrosis in patients.

The purpose of this preliminary study is to elucidate possible interrelationships amongst circulating microparticles, angiogenic and angiostatic factors in SSc patients that are predictive of interstitial lung disease and high values of right ventricular systolic pressure (RVSP) by trans-thoracic Doppler-echocardiography (TTE).

Methods: Nineteen cases with limited cutaneous SSc (lcSSc) and 11 cases with diffuse cutaneous SSc (dcSSc) were compared to 30 age- and sex-matched healthy controls. High resolution computed tomography was used to establish the diagnosis of interstitial lung disease and TTE was used to estimate RVSP and to diagnose putative pulmonary arterial hypertension (PAH). Plasma concentrations of circulating factors were assessed in patients and controls.

Results: Angiopoetin-2, endostatin, E-selectin and platelet microparticles were higher in lcSSc cases compared to healthy controls ($p<0.0001$, $p=0.0008$, $p=0.0003$, $p=0.0020$, respectively). Only endostatin was higher in dcSSc cases ($p=0.0020$). In a classification tree analysis, concentrations of soluble E-selectin of at least 44.7 ng/ml and 37.2 ng/ml were predictive of ILD and PAH, respectively, in lcSSc cases, whereas, endothelial microparticle levels higher than 96 per µl were predictive of ILD in dcSSc cases.

Conclusion: Scleroderma cases can be differentiated from healthy controls based on higher concentrations of the angiostatic factor endostatin. E-selectin was associated with lung involvement in lcSSc, whereas, high levels of endothelial microparticles were associated with ILD in dcSSc.

Keywords: Diffuse scleroderma; Limited scleroderma; Pulmonary fibrosis; Pulmonary hypertension; Systemic scleroderma

Abbreviations: ACA: Anti-Centromere Antibodies; ANA: Anti-Nuclear Antibodies; dcSSc: Diffuse Cutaneous Scleroderma; ELISA: Enzyme-Linked Immunosorbent Assay; EMP: Endothelial Microparticle; ILD: Interstitial Lung Disease; lcSSc: Limited Cutaneous Scleroderma; PAH: Pulmonary Arterial Hypertension; PAP: Pulmonary Arterial Pressure; PMP: Platelet Microparticle; RVSP: Right Ventricular Systolic Pressure; sAng-1: Soluble Angiopoietin 1; sAng-2: Soluble Angiopoietin 2; sE-selectin: Soluble E-selectin; sICAM-1: Soluble Inter-Cellular Adhesion Molecule-1; sL-selectin: Soluble L-selectin; SSc: Scleroderma (Systemic Sclerosis); sTie-2: Soluble Endothelial Cell Specific Tyrosine Receptor 2; sVCAM-1: Soluble Vascular Cell Adhesion Molecule 1; TTE: Trans-Thoracic Doppler-Echocardiography; VEGF: Vascular Endothelial Growth Factor; VEGF-R1: Receptor 1 for Vascular Endothelial Growth Factor

Introduction

Insufficient or absent angiogenesis are hallmarks of scleroderma (SSc) [1]. Microvascular change is an early manifestation of SSc followed by intimal proliferation and fibrosis of arterioles resulting in reduced blood flow and tissue ischemia [2]. This ongoing vasculopathy in the lungs presents clinically as pulmonary hypertension and characteristically precedes lung fibrosis [3].

With tissue hypoxia and vascular damage, vascular healing and new blood vessels are required [4]. Angiogenesis is dependent on a tight balance of angiogenic and angiostatic factors [5]. Different pro-angiogenic stimuli are present in SSc but an appropriate angiogenic response is lacking [6]. In a hypoxic environment, growth factors outweigh inhibitors with angiogenesis the result [7]. Vascular endothelial growth factor (VEGF) is a hierarchical upstream inducer of angiogenic cascade [8-11]. VEGF synthesis is paralleled by increased activation and apoptosis of endothelial cells. This is illustrated on a molecular level by biomarkers inclusive of inter-cellular adhesion molecule 1 (ICAM-1) and the vascular cell adhesion molecule 1(VCAM-1) [12]. Circulating microparticles regulate inflammation, affect cell proliferation and apoptosis [13]. They were shown in a previous study to be elevated in 10 dcSSc and 27 lcSSc patients combined compared to 15 healthy controls ($p<0.05$), suggesting that microparticles attenuate fibrotic disease manifestations [14].

A second set of regulatory molecules, the endothelial cell specific receptor tyrosine kinases Tie-1 and Tie-2, and their ligands angiopoietin-1 (Ang-1) and angiopoietin-2 (Ang-2), also regulate both vasculogenesis and angiogenesis [15]. Ang-1 is expressed by many cells including pericytes, smooth muscle cells and fibroblasts [16], while Ang-2 is expressed almost exclusively by endothelial

***Corresponding author:** Kevin J Keen, PhD, Department of Mathematics and Statistics, University of Northern British Columbia, 3333 University Way, Prince George, BC, Canada, Tel: 250-961-8301; E-mail: keenk@unbc.ca

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cells. Ang-1 mediated Tie-2 signaling is the adult default pathway to control vascular quiescence. Ang-2 antagonizes Ang-1 and responds to endothelial stimulation by several factors including VEGF, fibroblast growth factor-2, tumor necrosis factors and hypoxia [17]. Ang-2 may be the principal switch controlling the transition from resting to activated endothelium and facilitating inflammatory response [18].

Yu et al. [19] have demonstrated *in vivo* and *ex vivo* that E-selectin is required in endostatin-mediated anti-angiogenesis and confers endostatin sensitivity to nonresponsive human endothelial cells *in vitro*.

Tissue ischemia normally leads to up-regulation of angiogenic factors which, through the proliferation of endothelial cells, aids in vascular remodeling. Despite reduced organ perfusion in SSc, there is paradoxically no increase in angiogenic activity to compensate for this deficiency [20]. The more extensive and severe disease occurs in the diffuse subset. At least one-third of patients have clinically significant fibrosis and lung function impairment.

The purpose of this preliminary study is to elucidate in SSc patients possible interrelationships amongst circulating Microparticles and angiogenic and angiostatic factors that are predictive of lung fibrosis and high values of right ventricular systolic pressure (RVSP) by trans-thoracic Doppler-echocardiography (TTE).

Materials and Methods

Study population

Thirty cases with SSc and 30 healthy controls gave informed consent. Research was done in compliance with the Declaration of Helsinki and approved by the Research Ethics Boards of the University of British Columbia and the University of Northern British Columbia. All patients fulfilled the American College of Rheumatology Criteria for SSc and were classified as having limited cutaneous (lcSSc) or diffuse cutaneous (dcSSc) disease according to the criteria of LeRoy et al. [21]. The study protocol excluded cases or age- and sex-matched healthy controls with hypertension, known coronary artery disease, known cardiovascular disease, diabetes, lung disease or active infections at the time of phlebotomy. Cases with overlap syndromes or mixed connective tissue disease were excluded from the study. No recruited healthy control was excluded and the sole case excluded was a dcSSc male with hypertension.

Clinical variables

Disease duration was measured from the onset of the first non-Raynaud's phenomenon. Skin involvement was assessed using the modified Rodnan skin score (mRSS) at 17 different body areas and given a score from 0-3 for each area [22]. Disease severity for each patient was evaluated on a scale of 0-4 for each of nine organ systems using the Medsger scale [23] and then summed.

Pulmonary function was examined but only predicted forced vital capacity (FVC) and the predicted diffusing level of carbon monoxide (DLCO) are reported. Interstitial lung disease (ILD) was defined as the presence of ground glass opacities, fibrosis or honeycombing. It was determined from high resolution computed tomography (HRCT) [24].

Study patients underwent HRCT of the chest with slice thickness of 1.25 mm taken at an interval of 0.8 mm at 100 kV and 360 mA. The scanning was performed through the entire thorax with the subject in a supine position with breath holding in inspiration. The HRCTs were read by an experienced chest radiologist blinded to the clinical and lab data and reviewed by us. Ground glass opacity was defined as a hazy parenchymal opacity with preservation of bronchial and vascular margins. Fibrosis was defined as interlobular septal thickening, intralobular lines, traction bronchiectasis and traction bronchiolectasis. Honeycombing was defined as clustered air filled cysts with well defined walls. Formal scoring was not used in this study.

TTE was used for RVSP and Doppler flow measurement of the TR jet to calculate pulmonary arterial pressure (PAP). Pressures less than 35 mm Hg were considered normal. Echo Doppler studies have been shown to correlate well with right heart catheter studies [25], but tend to underestimate pulmonary artery pressure and cardiac output [26]. All clinical variables were recorded at time of plasma collection or within two months prior.

Microparticle concentrations

Blood samples were collected in buffered sodium citrate and centrifuged at 1 500 g for 15 minutes. The plasma was removed and spun at 12 700 g for 12 minutes. Without disturbing the pellet, the platelet free plasma (PFP) was removed and stored at -80°C. In brief, 100 ml of PFP was incubated (20 minutes) with 20 ml of anti-CD 106-FITC (BD Bioscience, Mississauga, Canada), 7.2 ml of anti-CD 144-PE (eBioscience, San Diego, California, USA), and 20ml of anti-CD41-PECy5 (BD Bioscience). Mouse immunoglobulin (Dako Canada, Burlington, Canada) was used as a negative control. Samples were then incubated with 10 µl of Annexin V-APC (BD Bioscience) and 100 ml of $CACl_2$ (5 mM) binding buffer for 15 minutes. Finally, 250 ml of binding buffer and 100 ml of Flow-Count counting beads (Beckman Coulter Canada, Mississauga, Canada) were added to each tube. Samples were placed on ice and analyzed immediately using a MoFlo high speed cell sorter (Beckman Coulter Canada). Microparticles were defined as having a size of 1 µm or less. With a forward- and side-scatter dot plot, the upper boundary of the analysis region was set using a 1.0 µm fluorescent microsphere (Polysciences, Warrington, Pennsylvania, USA). A 0.5 µm microsphere was used to ensure visualization of events of this size or smaller. Gated events were then interrogated for expression of labeled antibodies. Platelet microparticles (PMPs) were defined as $CD41^+$ MPs. Endothelial microparticles (EMPs) were defined as $CD41^-$ /$CD144^+$ MPs. To confirm EMPs, samples were further evaluated for the expression of activation ($CD106^+$) or apoptosis (Annexin V^+) markers (data not shown). Sample analysis was stopped at 50 000 MPs. The numbers of EMPs and PMPs per microliter were calculated as per the Flow-Count product insert (Beckman Coulter Canada).

Circulating factors

All plasma samples were aliquoted and stored at -80°C. Plasma levels of soluble Angiopoeitin-1 (sAng-1), Angiopoeitin-2 (sAng-2) and sTie-2 (lower case "s" prefix denotes soluble) were measured by commercially available quantitative colorimetric sandwich enzyme-linked immunosorbent assay (ELISA) kits following the manufacturer's protocol (R&D Systems, Minneapolis, Minnesota, USA). Endostatin was measured by a sandwich ELISA (R&D Systems, Minneapolis, Minnesota, USA). VEGF, soluble intercellular adhesion molecule one (sICAM-1), soluble vascular cell adhesion molecule one (sVCAM-1), sE-selectin and sL-selectin were measured using Searchlight Custom Arrays (Pierce Searchlight Products, Thermo Fisher Scientific Woburn, Massachusetts, USA) and analyzed using a 16-bit Searchlight CCD imaging system (Pierce Biotechnology, Rockford, Illinois, USA).

Statistical analysis

Estimates of location are reported as a mean ± SE. Given small

sample sizes and non-normality for most factors, nonparametric methods were used for comparisons. Statistical calculations and graphing were done using release 2.11.1 of the R statistical software system [27]. The *stats* package in R was used to calculate the exact *P* values for the Wilcoxon test of no shift in location for matched case-control pairs. The *coin* package in R was used to calculate the exact *P* values for the Mann-Whitney test for location with two independent random samples [28]. Classification trees for ILD and PAH as an exploratory investigation were produced using the *rpart* package [29]. Because of small sample sizes, the accuracy of correct classification for each tree was assessed with the delete-one jackknife. Caution in interpretation of the accuracy estimates for classification trees is advisable because of the small sample sizes.

Results

Demographics and clinical features of patients

Blood samples were available for this preliminary study of 19 patients diagnosed with lcSSc and 11 with dcSSc (Table 1). No gender difference in age with the healthy controls was detected for lcSSc patients (p=0.0917) or for dcSSc patients (p=0.6970). Combining genders, patients diagnosed with lcSSc had a mean (±SE) age of 58.53 ± 2.50 years (range: 36-74 years) while patients diagnosed with dcSSc had a mean age of 49.64 ± 3.17 years (range: 30-65 years). The difference in median age between lcSSc and dcSSc is statistically significant (p=0.0399).

There was no difference in location of the distribution of maximum disease severity scores between women and the single man with lcSSc (p=0.8889) or amongst the female and male dcSSc patients (p>0.9999). Combining genders, patients diagnosed with lcSSc had a mean (±SE) maximum disease severity index of 7.00 ± 0.78 (range: 2-16). Patients diagnosed with dcSSc had a mean disease severity index of 7.18 ± 0.91 (range: 2-11). There is no statistically significant difference between lcSSc and dcSSc cases with respect to location of the disease severity index (p=0.6638) as a consequence of aggressive clinical management or referral bias associated with a tertiary care clinic, or both.

No gender difference in disease duration was detected for lcSSc (p=0.9474) or for dcSSc (p=0.8485) patients. Combining genders, patients diagnosed with lcSSc had a mean (±SE) disease duration of 10.68 ± 2.68 years (range: 1-43 years) while patients diagnosed with dcSSc had a mean disease duration of 2.91 ± 0.48 years (range: 2-11 years). Disease duration is significantly shorter (p=0.0381) in dcSSc cases compared to lcSSc as expected.

No gender difference in mRSS was detected for either lcSSc (p=0.2632) or dcSSc (p=0.7576) cases. Mean (±SE) mRSS was 9.05 ±1.29 (range: 1-20) in lcSSc cases and 22.82 ± 2.82 (range: 4-36) in dcSSc cases. Skin score is worse in dcSSc cases compared to lcSSc cases (p=0.0002).

With regard to auto antibodies, dcSSc and lcSSc have distinctly different profiles for anti-centromere antibodies (ACA), anti-nuclear antibodies (ANA) and anti-topoisomerase antibodies (Scl-70) (Table 1).

On the basis of the preceding statistical analyses, decisions were made to disregard gender but treat limited and diffuse forms of SSc as distinct.

Comparisons of circulating factors among cases and healthy controls

There are insufficient data to estimate the 78 variances and

Variable	Disease	Category	Estimate	Count
Proportion	lcSSc	Female: Male	18.0:1	18:1
	dcSSc	Female: Male	1.2:1	6:5
Severity Index	lcSSc		7.00 ± 0.78	18
	dcSSc		7.18 ± 0.91	11
Modified Rodnan Skin Score	lcSSc		9.05 ± 1.29	19
	dcSSc		22.82 ± 2.82	11
Age	lcSSc		58.53 ± 2.50 yr	19
	dcSSc		49.64 ± 3.17 yr	11
Duration	lcSSc		10.68 ± 2.68 yr	19
	dcSSc		2.91 ± 0.48 yr	11
Lung				
ILD	lcSSc		47.4 ± 11.5 %	19
	dcSSc		63.6 ± 14.5 %	11
Putative PAH	lcSSc		47.4 ± 11.5 %	19
	dcSSc		36.4 ± 14.5 %	11
FVC Predicted	lcSSc		86.6 ± 4.5 %	18
	dcSSc		73.4 ± 6.5 %	11
DLCO Predicted	lcSSc		63.1 ± 4.9 %	19
	dcSSc		64.8 ± 8.3 %	10
Telangiectasia	lcSSc		17.6 ± 9.2 %	17
	dcSSc		20.0 ± 12.6 %	10
Digits				
Raynaud's	lcSSc		100 %	18
	dcSSc		100 %	11
Active digital ulcers	lcSSc		35.3 ± 11.6 %	17
	dcSSc		30.0 ± 14.5 %	10
No. of digits with Active digital ulcers	lcSSc		0.71 ± 0.28	17
	dcSSc		0.88 ± 0.48	9
Contracture of phalanges	lcSSc		41.2 ± 11.9 %	17
	dcSSc		63.6 ± 14.5 %	11
Autoantibodies				
ACA positive	lcSSc		15.8 ± 8.4%	19
	dcSSc		0 %	11
ANA positive	lcSSc		84.2 ± 8.4%	19
	dcSSc		90.1 ± 8.7%	11
Scl-70 positive	lcSSc		15.8 ± 8.4%	19
	dcSSc		72.7 ± 13.4%	11
Treatment				
Methotrexate	lcSSc		0 %	19
	dcSSc		18.2 ± 11.6 %	11
Cyclophosphamide	lcSSc		0 %	19
	dcSSc		45.5 ± 15.0 %	11

Except where indicated otherwise, values are the mean ± SE

Table 1: Demographics.

covariances for the 12 circulating factors. It is reasonable to assume dependencies among the circulating factors, so the Bonferroni correction for 24 independent tests with an overall 0.05 level of significance is conservative in the case of unassessed dependencies. Except for the selectins and VEFG Receptor-1 (VEGF-R1), the distributions of factors and microparticles are not normally distributed (Supplementary Table 1).

The lcSSc and dcSSc cases are comparable with the following exceptions. The serum concentration of sAng-2 in lcSSc is statistically significantly greater than in the matched controls ($p<0.0001\div2=0.00005$) (Table 2). This is consistent with an activated endothelium facilitating inflammatory response in lcSSc patients. Endostatin levels are higher in both sets of SSc cases compared to their paired controls (Table 2). sE-selectin levels are higher in lcSSc patients compared to their controls ($p=0.0003\div2=0.0002$) but not in dcSSc patients ($p=0.0840\div2=0.0420$). Amongst the cell adhesion molecules assessed, sE-selectin appears to be the most promising. The only statistically significant result involving microparticles is the lower level of PMPs in lcSSc patients ($p=0.0020\div2=0.0010$) compared to controls, although the mean for dcSSc patients is even lower.

Overall, the dramatic differences by p-value are higher concentrations of sAng-2, endostatin, sE-selectin, and low PMP in 19 lcSSc patients and higher concentrations of endostatin in 11 dcSSc cases compared to controls.

Comparisons of circulating factors relating to ILD

The prevalence of ILD is 47.4 per cent and 63.6 per cent in the lcSSc and dcSSc cases, respectively (Table 1). In Table 3, the only two circulating factors with concentrations that appear to be different in cases with ILD are sE-selectin which was higher in lcSSc cases ($p=0.0535\div2=0.0268$) and EMPs which were higher in dcSSc cases ($p=0.0121\div2=0.0061$).

For a classification tree [30] with the 12 circulating factors for ILD in lcSSc cases, the factors in order of appearance from the top of the tree to the bottom are sE-selectin, sAng-1, and sAng-2 (Figure 1A). The estimate of accuracy of correct classification by the classification tree method is 47.4 per cent. sAng-2 came to our attention in the comparison of lcSSc cases with their controls (Table 2). That sE-selectin has entered the model for the classification tree for lcSSc is not a surprise because of its role in inflammation. The angiopoeitins, especially Ang-2, are important regulators of the inflammatory response.

	Control	lcSSc		dcSSc	
	Estimate	Estimate	p-value	Estimate	p-value
sAng-1	9 207 ± 808	10 622 ± 1 662	0.7381	13 889 ± 2 201	0.1934
sAng-2	1 864 ± 122	4 944 ± 941	**<0.0001**	3 982 ± 498	0.0039
sTie-2	30.8 ± 1.0	37.7 ± 2.8	0.0082	37.7 ± 2.1	0.2324
Endostatin	143 ± 12	261 ± 33	**0.0008**	259 ± 32	**0.0020**
VEGF	195 ± 20	290 ± 74	0.5153	270 ± 66	0.6953
VEGF-R1	170 ± 10	173 ± 20	0.8288	178 ± 20	0.3750
sE-selectin	18.2 ± 1.2	34.0 ± 3.9	**0.0003**	30.5 ± 3.1	0.0840
sL-selectin	906 ± 35	961 ± 61	0.2753	755 ± 57	0.0273
sICAM-1	233 ± 12	314 ± 49	0.1956	316 ± 29	0.0840
sVCAM-1	1 152 ± 57	1 443 ± 164	0.2101	1 205 ± 115	0.6250
EMP	111 ± 13	116 ± 21	0.6794	200 ± 50	0.3223
PMP	300 ± 79	161 ± 51	**0.0020**	120 ± 24	0.4316

Values are the mean ± SE. Unit of measurement is pg/ml for sAng-1, sAng-2, VEGF, and VEGF-R1. Unit of measurement is ng/ml for sTie-2, Endostatin, sE-selectin, sL-selectin, sICAM-1, and sVCAM-1 Microparticles are measured in counts per µl. The p-values are for paired testing of no location shift between cases with matched controls by the exact Wilcoxon signed rank test. One male dcSSc case did not match a female control and so both were dropped from the Wilcoxon signed rank tests The p-values in bold are less than 0.05/24 = 0.0021

Table 2: Comparison of mean concentrations of circulating factors among cases and controls.

	lcSSc			dcSSc		
ILD	Yes	No		Yes	No	
			p-value			p-value
sAng-1	13 076 ± 2 823	8 413 ± 1 575	0.9682	14 622 ± 2 965	12 607 ± 3 571	0.9273
sAng-2	6 633 ± 1 798	3 423 ± 488	0.2428	4 569 ± 683	2 954 ± 294	0.3152
sTie-2	36.8 ± 3.9	38.4 ± 2.0	0.1333	35.1 ± 2.1	42.2 ± 6.8	0.6485
Endostatin	296 ± 54	230 ± 38	0.4967	279 ± 50	225 ± 20	0.9273
VEGF	418 ± 146	176 ± 29	0.0947	307 ± 102	207 ± 38	0.7879
VEGF-R1	191 ± 36	157 ± 20	0.7197	178 ± 33	178 ± 7	0.7879
sE-selectin	43.3 ± 6,2	25.7 ± 3.3	0.0535	33.7 ± 4.1	25.2 ± 3.6	0.3152
sL-selectin	960 ± 101	963 ± 78	0.7197	673 ± 44	899 ± 111	0.1091
sICAM-1	343 ± 104	288 ± 22	0.4002	326 ± 37	298 ± 54	0.9273
sVCAM-1	1 616 ± 306	1 287 ± 146	0.5490	1 281 ± 172	1 072 ± 91	0.5273
EMP	97 ± 23	132 ± 35	0.6607	277 ± 63	66 ± 9	0.0121
PMP	120 ± 32	198 ± 94	0.8421	146 ± 32	76 ± 27	0.1636

Values are the mean ± SE. Unit of measurement is pg/ml for sAng-1, sAng-2, VEGF, and VEGF-R1. Unit of measurement is ng/ml for sTie-2, Endostatin, sE-selectin, sL-selectin, sICAM-1, and sVCAM-1. Microparticles are measured in counts per µl. The *P* values are for paired testing of no difference of location between cases with and without lung fibrosis within each disease type by the exact Mann-Whitney test

Table 3: Comparison of mean concentrations of circulating factors by ILD and disease type.

Mean EMP concentrations are more than 4 times higher in dcSSc cases with ILD compared to dcSSc cases without ILD (p=0.0121÷2=0.0061) (Table 3). So it is not a surprise to have EMP as the circulating factor at the top of the classification tree for dcSSc patients (Figure 1B). The estimate of accuracy of correct classification by the classification tree method is 72.7 per cent. Six patients with ILD are picked out at the first branch with a concentration greater than or equal to a count of 95 per µl. The remaining dcSSc patient with ILD is picked out at the second branch with an sAng-1 concentration less than 7.8 ng/ml.

The classification trees for lcSSc and dcSSc are distinctly different for ILD. Low concentrations of sE-selectin and sAng-1 with a high concentration of sAng-2 appear to predict an absence of ILD for lcSSc cases, whereas, a low EMP concentration with a high sAng-1 concentration appear to predict an absence of ILD for dcSSc cases. For either lcSSc or dcSSc, the angiopoetins seem to be involved in regulating endothelial activation and inflammation but ILD in lcSSc appears to be a consequence of inflammation associated with E-selectin. In contrast, ILD in dcSSc appears to be characterized by large numbers of circulating endothelial microparticles.

Comparisons of circulating factors relating to pulmonary hypertension

The prevalence of putative PAH is 47.4 per cent and 36.4 per cent in the lcSSc and dcSSc cases, respectively (Table 1). The concordance rates for ILD and high RVSP are 68.4 per cent and 54.5 per cent for lcSSc and dcSSc cases, respectively, so there is not complete overlap of these two clinical conditions (Table 4). No factor appears by the Bonferroni correction to be significantly different with respect to a difference in location whether RVSP ≥ 35 mm Hg, or not, for either lcSSc or dcSSc (Table 5).

The classification tree for putative pulmonary hypertension amongst the 19 lcSSc cases starts with sE-selectin at the top (Figure 2A). The estimate of accuracy of correct classification by the classification tree method 57.9 per cent. Six of the high lcSSc cases have an sE-selectin concentration of at least 37 ng/ml. A sequence of two binary splits at the second branch proceeds through PMP and sICAM-1 to separate the remaining 3 cases with TTE RVSP ≥ 35 mm. PMP first attracted attention in comparison tests with controls (Table 2). Generally, lcSSc cases with low sE-selectin, PMP and sICAM-1 concentrations will have TTE RVSP < 35 mm.

The classification tree for determining putative pulmonary hypertension amongst the dcSSc cases is simple (Figure 2B): 3 dcSSc cases with putative pulmonary hypertension have a VEGF-R1 concentration of 206 pg/ml or higher. The mean (±SE) VEGF-R1 concentration for dcSSc cases is 178 ± 20 pg/ml. The remaining dcSSc case with putative pulmonary hypertension separates from those without at the second branch with an EMP count less than 56 particles per µl. The estimate of accuracy of correct classification by the classification tree method 63.6 per cent.

Comparing classification trees suggests that the potent inflammatory agent E-selectin is associated with both ILD and high TTE RVSP in lcSSc (Figure 1A and Figure 2A). This is not repeated in the classification trees for dcSSc (Figures 1B and Figure 2B) in which high EMP concentrations are associated with ILD and TTE RVSP lower than 35 mm. So while endothelial microparticles appear responsible for ILD in dcSSc cases they are protective against pulmonary hypertension. The implication is that lcSSc reaches the endpoints of lung fibrosis and pulmonary hypertension through a different biochemical pathway than dcSSc.

Discussion

With regard to the literature, Blann et al. [31] reported mean concentrations of soluble L-selectin in serum for dcSSc cases (755 ±57 ng/ml), lcSSc cases (961 ± 61 ng/ml) and controls (906 ±35 ng/m). Shimada et al. [32] reported sample means in serum. The estimates

	lcSSc		dcSSc	
ILD	**Present**	**Absent**	**Present**	**Absent**
Putative PAH				
Present	6	3	3	1
Absent	3	7	4	3

Table 4: Distribution of ILD and PAH status.

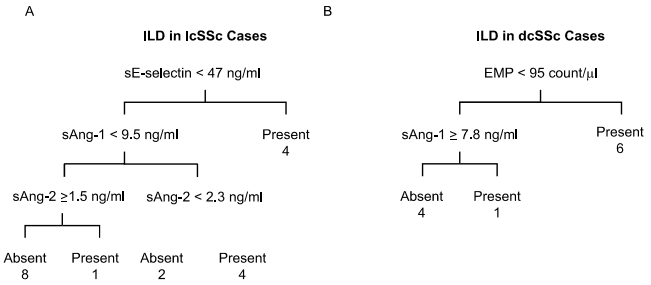

Figure 1: Classification trees for determining presence or absence of ILD among lcSSc (A) and dcSSc (B) cases. If a statement is true then the branch to the left is taken.

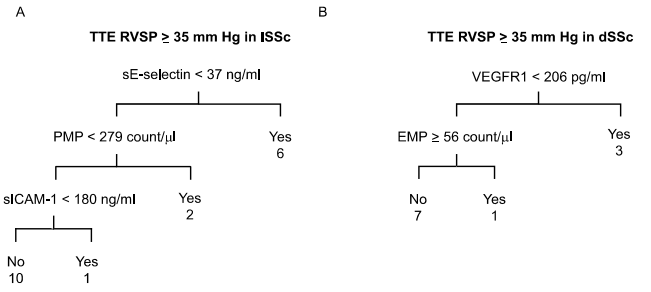

Figure 2: Classification tree for determining putative pulmonary hypertension among lcSSc and dcSSc cases. If a statement is true then the branch to the left is taken.

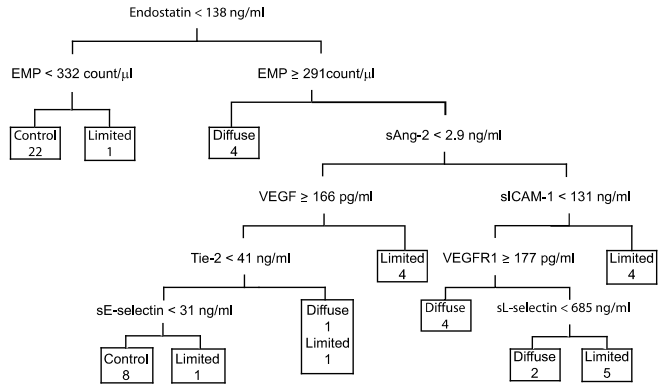

Figure 3: Classification tree for sorting amongst controls, lcSSc and dcSSc cases. If a statement is true then the branch to the left is taken.

	lcSSc			dcSSc		
Putative PAH	**Present**	**Absent**		**Present**	**Absent**	
			p-value			**p-value**
sAng-1	11 770 ± 2,901	9 588 ± 1 737	0.6607	14 975 ± 4 270	13 269 ± 2 701	0.6485
sAng-2	7 360 ± 1,641	2 770 ± 325	0.0076	4 199 ± 1 043	3 858 ± 576	0.9273
sTie-2	41.0 ± 2.6	34.7 ± 3.0	0.1128	36.4 ± 2.2	38.4 ± 4.4	0.7879
Endostatin	308 ± 51	219 ± 39	0.1333	256 ± 49	261 ± 46	>0.9999
VEGF	431 ± 145	163 ± 23	0.0435	406 ± 165	193 ± 30	0.1091
VEGF-R1	189 ± 36	159 ± 21	0.5490	234 ± 32	146 ± 18	0.0424
sE-selectin	42.7 ± 6.7	26.3 ± 2.8	0.0535	31.1 ± 7.7	30.2 ± 2.8	0.9273
sL-selectin	948 ± 105	973 ± 73	0.5490	814 ± 48	721 ± 86	0.1636
sICAM-1	388 ± 95	248 ± 30	0.2775	335 ± 36	305 ± 42	0.6485
sVCAM-1	1 844 ± 283	1 082 ± 856	0.0101	1,157 ± 104	1 232 ± 177	0.7879
EMP	86 ± 23	142 ± 33	0.2775	150 ± 74	229 ± 68	0.6485
PMP	209 ± 107	118 ± 20	0.6607	83 ± 9	142 ± 36	0.3152

Values are the mean ± SE. Unit of measurement is pg/ml for sAng-1, sAng-2, VEGF, and VEGF-R1. Unit of measurement is ng/ml for sTie-2, Endostatin, sE-selectin, sL-selectin, sICAM-1, and sVCAM-1. Microparticles are measured in counts per µl. The p-values are for paired testing of no difference of location between cases with and without TTE RVSP ≥ 35 mm Hg within each disease type by the exact Mann-Whitney test signed rank test

Table 5: Mean concentrations of circulating factors by disease type and putative PAH status.

reported in this study are for soluble L-selectin in plasma and thus are not comparable with those from either Blann et al. [31] or Shimada et al. [32].

Supplementary Table 2 provides a comparison of estimates of location for VEGF in SSc cases and controls with those of Riccieri et al. [33], Papaioannou et al. [34], Guiducci et al. [14], Hummers et al. [35], Del Papa et al. [36] and Distler et al. [37]. Due to differences in methods it is not possible to perform hypothesis tests between any of these estimates and those of this study. Likewise, we cannot compare our estimate of median VEGF concentration (316 pg/ml, range: 72-1470 pg/ml) for SSc cases with putative pulmonary hypertension and those without (162 pg/ml, range: 92-382 pg/ml) with estimates of Papaioannou et al. [34]. However, these results and ours are trending to show VEGF concentration to be higher in plasma, or serum, for SSc cases compared to controls and as well higher in SSc patients with a TTE RVSP of 35 mmHg or higher compared to SSc cases with TTE RVSP lower than 35 mmHg.

The literature review for endostatin concentrations revealed no consistency (Supplementary Table 3) [35,37,38]. Statistical tests of comparison are not possible because of the differences in assay methods.

Two articles were found that considered microparticle concentrations in SSc patients. The results of Nomura et al. [39] are not comparable to our results because they used the monoclonal antibody GPIX. The later article by Guiducci et al. [14] counted the number of $CD144^+$ microparticles in contrast to the mass of $CD41^-$ /$CD144^+$ microparticles reported by us.

There is evidence for greater expression of E-selectin in neutrophil co-cultures from sera of SSc cases compared to controls (p=0.00004) [40]. Our study consistently shows lower ratios for cases to controls compared to the literature for sVCAM-1 and sICAM-1 but not sE-selectin (Supplementary Table 4) [36,39,41].

We found plasma levels of sAng-1, sAng-2, and sTie-2 to be higher in SSc cases compared to sex- and age-matched healthy controls. Riccieri et al. [33] examined only sAng-2 in plasma with a difference assay method but also come to the same conclusion (Supplementary Table 5). However, Michalska-Jakubus et al. [42] in considering only sAng-1 and s-Ang-2 in serum found the opposite to be true. Noda et al. [43] found no difference between SSc cases and controls with respect to the concentration of sTie-2 in serum. Nevertheless, ILD in our lcSSc cases is associated with high levels of sAng-1 and sAng-2 (Figure 1). But sAng-1, sAng-2, and sTie-2 are not implicated in putative PAH (Figure 2).

This preliminary study is limited by being cross-sectional on a small number of patients, in measuring only plasma levels of circulating factors and estimating PAP by TTE. Lacking are measurements of profibrotic factors, such as Interleukin 6. Data collection is ongoing.

In attempting to distinguish healthy controls from lcSSc and dcSSc cases, 22 controls can be separated from all but one lcSSc case based upon a cut-off of endostatin concentration below138 ng/ml in a classification tree (Figure 3). In distinguishing the remaining 8 controls from the cases, this is achieved at the last node on the classification tree by an sE-selectin concentration less than 31 ng/ml (Figure 3). This is consistent with the demonstration by Yu et al. [19] *in vivo* and *ex vivo* that E-selectin is required in endostatin-mediated antiangiogenesis and confers endostatin sensitivity to nonresponsive human endothelial cells *in vitro*. The interaction of VEGF and its receptor VEGF-R1 requires further study as does the potential role of L-selectin.

In summary, our classification tree analysis in this preliminary report provides support for a role of the sAng-1/sAng-2/sTie-2 system with respect to ILD in both lcSSc and dcSSc, however, more research is needed. For the limited form of disease, ILD and putative pulmonary hypertension are associated with high concentrations of the potent pro-inflammatory agent sE-selectin, whereas, the diffuse form finds endothelial microparticles more strongly connected with ILD but not putative pulmonary hypertension. Higher levels of microparticles have been associated with promoting adhesion in vascular disorders [44].

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