Evaluation of endothelial dysfunction and clinical events in patients with early-stage vasculopathy in limited systemic sclerosis

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ABSTRACT

Objective. *Limited cutaneous systemic sclerosis (lcSSc) is characterised by vasculopathy contributing to vascular apoptosis, structural and functional changes. The aim of this study was to investigate parameters of endothelial dysfunction and their association to clinical events in lcSSc patients with early-stage vasculopathy.*

Methods. *Patients with lcSSc and early-stage vasculopathy defined as absent pre-existing pulmonary arterial hypertension (PAH), digital ulcers, and symptomatic cardiovascular diseases were recruited together with age-, race- and sex-matched controls with primary Raynaud's phenomenon. All subjects underwent measurements of flow-mediated (FMD) and nitroglycerine-mediated dilation (NMD), pulse-wave analysis, and biochemical analysis, including arginine, homoarginine, citrulline, ornithine, asymmetric dimethylarginine (ADMA), symmetric dimethylarginine (SDMA), and endothelial microparticles (EMP). Clinical events, including EUSTAR index, sicca symptoms, microvascular, skin, renal, gastrointestinal, and pulmonary involvement, were recorded by medical history, physical examination, laboratory parameters, disease-specific questionnaire, electrocardiogram, diagnostic imaging and spirometry.*

Results. *38 patients with lcSSc and 38 controls were included after screening for eligibility. There was no difference in FMD (p=0.775), NMD (p=0.303), aortic pulse-wave velocity (p=0.662) or in augmentation index (p=0.600) between patients with lcSSc and controls. Higher values of ADMA (p=0.030), SDMA (p=0.025) and borderline significantly higher values for CD31+/CD42b- EMP (p=0.062) were observed in lcSSc patients, also with positive correlations between those parameters. ADMA, SDMA and CD31+/CD42b- were correlated with subclinical PAH, nephropathy and capillary changes.*

Conclusion. *Selected parameters of endothelial dysfunction contribute to clinical events in lcSSc patients with early-stage vasculopathy and endothelial dysfunction seems to be primarily present in microvasculature, while its impact on macrovascular changes in lcSSc is still indistinct.*

Introduction

Systemic sclerosis (SSc) is characterised by vasculopathy, autoimmune activation and progressive fibrosis affecting the skin and internal organs. SSc can be classified into three subtypes: diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) and SSc without skin involvement, with differences in pathogenesis, clinical manifestations and outcome between those subtypes (1, 2). SSc-related vasculopathy, including endothelial dysfunction, may occur at an early stage, especially in lcSSc, affecting predominately microcirculation although macrovascular damage has also been reported (3-6). Complex interactions between different cell types may promote vascular apoptosis, lymphocyte migration, and fibroblast proliferation leading subsequently to clinical manifestations of lcSSc including Raynaud's phenomenon, capillary changes, digital ulcers and pulmonary arterial hypertension (PAH) (4, 7). Numerous parameters have been implicated as markers of endothelial dysfunction. Flow-mediated dilation (FMD) and pulse-wave velocity (PWV) have been thoroughly evaluated in atherosclerotic diseases while altered FMD and PWV was established in patients with SSc too (5, 6, 8-10).

Additionally, several biochemical parameters including compounds of the arginine metabolism, like asymmetric dimethylarginine (ADMA) or symmetric dimethylarginine (SDMA), and endothelial microparticles (EMP) mediate endothelial dysfunction by interacting with nitric oxide (NO) metabolism, vascular inflammation, and platelet function (11-14). In SSc, ADMA and EMP may be involved in the development of PAH and microangiopathic changes (15-18).

Although various data about endothelial dysfunction in SSc are available, they have some limitations. Several studies evaluated parameters of endothelial dysfunction without a control cohort or used controls without age- and sex-matching (15, 18-20). Additionally, a combined subject population including patients with lcSSc and dcSSc, despite pathogenetic differences between these subtypes, were commonly used or SSc subjects with present or experienced vasculopathy-mediated complications, like digital ulcers or PAH, were included (3, 16, 21, 22).

The aim of this study was to investigate endothelial dysfunction, assessed by parameters of vascular reactivity and arterial stiffness, EMP, compounds of the arginine metabolism and the association of these parameters between each other as well as between clinical events in lcSSc patients with early-stage vasculopathy.

Material and methods

Study design and patient cohort

Patients with known lcSSc diagnosed between 1997 and 2019 were screened for study inclusion and invited to participate. For every lcSSc subject, one age- (± 3 years), race- and sex-matched control with known primary Raynaud's phenomenon diagnosed between 2006 and 2019 was included after screening for study inclusion. Inclusion criterion for the group of patients with lcSSc was a present lcSSc diagnosed by the ACR/EULAR criteria (2). Exclusion criteria for the group of patients with lcSSc and controls were age <18 years, presence of dcSSc or other connective tissues diseases, pre-existing or existing PAH, digital ulcers, endoscopic approved reflux, diabetes mellitus or symptomatic atherosclerotic cardiovascular diseases (angina pectoris, myocardial infarction, stroke, intermittent claudication and/or rest pain of the lower or upper limb), recent pregnancy or malignancies, acute infections at time of enrolment, and current intake (<24 hours) of prostanoids, calcium channel blockers, phosphodiesterase-5 inhibitors or endothelin-receptor inhibitors.

Between April 2019 and February 2020, parameters of endothelial dysfunction and clinical parameters were investigated. Primary endpoint was the difference of FMD between patients with lcSSc and controls. Secondary endpoints were present FMD response <7%, differences of the remaining parameters of endothelial dysfunction and the association of endothelial dysfunction to clinical events within patients with lcSSc. After signing the informed consent, blood sampling and urinalysis for biochemical analysis were obtained followed by medical history and completion of questionnaires. Subsequently, electrocardiogram (ECG), pulse-wave analysis, physical examination including modified Rodnan Skin Score (mRSS), assessment of FMD and nitroglycerine-mediated dilation (NMD), transthoracic right heart echocardiogram, digital acral plethysmography and nailfold videocapillaroscopy (NVC) were performed. Additionally, spirometry with diffusing capacity of the lung for carbon monoxide (DLCO) was performed within 2 months after study visit. Measurements of FMD, NMD, pulse-wave analysis, laboratory and clinical parameters, except for spirometry and DLCO, were performed in the morning between 7:00–11:00 am after an overnight fast in a temperature-controlled (22–24°C) and quiet room.

Vascular reactivity and arterial stiffness

All FMD and NMD measurements were performed by the same trained technician according to the guidelines by Corretti *et al*. (23) using a linear array transducer with 8-13MHz (Siemens ACUSON S2000™, Siemens Healthcare Corp., Erlangen, Germany). After a 5-minutes rest in supine position, a blood pressure cuff was placed below the antecubital fossa on the forearm and the brachial artery was examined in a longitudinal plane between 1-5 centimetres above the antecubital fossa. Three end-diastolic diameters between two intimal layers were measured ECG-gated during image acquisition in a one-centimetre-long segment of the brachial artery. Afterwards, the cuff was inflated 50mmHg above the resting systolic pressure for 5 minutes and then deflated. The post-ischaemic diameter of the brachial artery was measured 45 seconds after cuff release. FMD was defined as the change in post-ischaemic diameter as a percentage of the baseline diameter. After a rest of 20 minutes, NMD representing the endothelium-independent vascular response was performed. Diameter of the brachial artery was recorded similarly to the technique described for FMD before and three minutes after sublingual administration of 0.4mg nitroglycerine spray. Values of FMD <7% and NMD <15.6% were defined as pathologic accordingly to proposed reference values (24, 25).

Aortic PWV and augmentation index (Aix) were measured and calculated by automated analysis via the oscillometric device Mobil-O-Graph® (I.E.M. Mobil-O-Graph, I.E.M., Stolberg, Germany). After taking a resting ECG, size-adjusted cuff was placed on the right upper arm about 2–4 centimetres above the ante-cubital fossa in supine position and subsequent pulse-wave analysis was performed. Patients were requested not to speak and not to move over the whole pulse-wave analysis. Pathologic PWV was defined as >10m/s (9).

Biochemical analyses

Fasting blood samples for evaluation of arginine, homoarginine, citrulline, ornithine, ADMA, and SDMA as parameters of the arginine metabolism, EMP, N-terminal prohormone of brain natriuretic peptide, uric acid and kidney function were obtained with a needle of at least 21 gauges. Additionally, fasting urinalysis for the measurement of urine protein/creatinine, albumin/creatinine, α1-microglobulin/creatinine, β2-microglobulin/creatinine ratio and immunoglobulin G was performed.

Blood sample for measurement of parameters of the arginine metabolism were centrifuged at 4000g for 10 minutes at 15°C temperature within 1 hour after blood sampling obtainment. The supernatant was collected, divided into aliquots of 1ml and were stored at -80°C until final analysis in August 2020 by high-performance liquid chromatography with solid phase extraction and precolumn derivatisation firstly described by Teerlink *et al*. (26) with slight modifications (27). Arginine/ADMA, arginine/SDMA, homoarginine/ADMA, homoarginine/SDMA, arginine/citrulline and global arginine bioavailability (GAB) ratio, defined as ratio of arginine over ornithine plus citrulline, were calculated by division of the respective parameters.

Measurement for EMP were performed according to the recommendations for the analysis of extracellular vesicles published by Cossarizza *et al*. (28). Blood sample was collected without venous stasis in 5ml citrate tubes after discarding the first 2ml of blood and kept upright. The plasma was subsequently centrifuged at 2.500g for 15 minutes at room temperature to obtain platelet-poor plasma within 1 hour after blood sampling obtainment. 1ml of the supernatant was centrifuged again at 13.000g for 15 minutes at room temperature to obtain platelet-free plasma. The supernatant was collected and divided into aliquots of 0.1ml, which was snap-frozen in liquid nitrogen and stored at -80°C until further analysis in November 2020. A platelet-free plasma aliquot was thawed in a water bath at 37°C and immediately processed for fluorescence-activated cell sorting staining. 25µl of platelet-free plasma was incubated with fluorescein-isothiocyanate-labelled lactadherin (CellSystems, Troisdorf, Germany) and fluorochrome-labelled anti-human CD31 and CD42b antibodies (Biolegend, San Diego, USA) for 2 hours at 4°C in the dark. The corresponding fluorochrome-labelled isotype antibodies were used as controls. The samples were diluted 1:25 with 0.22µm filtered phosphate buffered saline prior to flow cytometric analysis. EMP were identified as CD31+/CD42b- events (13). A microparticle gate was established using fluorescent 1µm silica beads (Kisker Biotech, Steinfurt, Germany) for size calibration. Furthermore, immunohistochemical staining for CD51, CD54, CD62E, CD105, and CD144 were used to quantify CD51+/CD42b-, CD54+/CD42b-, CD62E+/CD42b-, CD105+/CD42b-, and CD144+/CD42b- EMP.

Urinalysis and blood samples of the remaining laboratory parameters were measured at a single central lab in sera and plasma of the patients.

Clinical events

Clinical events of lcSSc were defined as potential signs of PAH and lung fibrosis, renal, microvascular, skin and gastrointestinal involvement, as well as sicca symptoms. Additionally, disease severity was evaluated by the revised EUSTAR index (29). Disease-specific parameters defining clinical events are shown in Table I.

After obtaining blood samples, medical history and UCLA SCTC GIT 2.0 questionnaire were completed (30). Subsequently, ECG (GE MAC 1200 ST, General Electric Germany Ltd., Frankfurt, Germany) was taken after 5-minutes rest in supine position followed by physical examination including mRSS (31). Transthoracic right heart echocardiogram was conducted using microconvex array transducer with 1-4MHz (Siemens ACUSON S2000™, Siemens Healthcare Corp, Erlangen, Germany) in supine position after assessment of FMD followed by digital acral plethysmography of all fingers (ELCAT Vasoquant VQ4000, ELCAT Ltd, Wolfratshausen, Germany). Sub-

Table I. Clinical events and disease-specific parameters defining clinical events in lcSSc.

PAH	Microvascular involvement
Potential signs of PAH	Impaired acral perfusion
Recurrent dyspnea	Early pattern
Right axis deviation	Active pattern
P pulmonale	Late pattern
Right branch block	Giant capillaries
QTc prolongation >450ms	Microhaemorrhages
Basal right ventricle diameter >42mm	Capillary ramifications
Inferior cava vein diameter >21mm	Capillary loss
Estimated right atrial pressure >15mmHg	Capillary oedema
RV/LV ratio >1.0	Disorganisation of microvascular array
TAPSE <20mm	Bushy capillaries
Tricuspid regurgitation velocity >2.9m/s	CSURI
NT-proBNP >150pg/ml	MES
Predicted single breath DLCO <60%	
DETECT score	Gastrointestinal involvement
DETECT score step 1	UCLA SCTC GIT 2.0 total
DETECT score step 2	
Lung fibrosis	Sicca symptoms
Predicted FVC%	Feeling of xerostomia and/or xerophthalmia
Predicted FEV1%FVC	
Predicted RV%	EUSTAR index
Predicted TLC%	EUSTAR index ≥ 2.5
Predicted single breath DLCO%	
Skin involvement	Renal involvement
mRSS	GFR
Telangiectasia	Creatinine
Calcinosis cutis	Protein/creatinine ratio
Puffy finger	Albumin/creatinine ratio
Sclerodactyly	Urine immunoglobulin G
Acral necrosis	α1-microglobulin/creatinine ratio
Tendon friction rub	β2-microglobulin/creatinine ratio

CSURI: capillaroscopic skin ulcer risk index; DLCO: diffusing capacity; EUSTAR: European Scleroderma Trials and Research Group; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; GFR: glomerular filtration rate; MES: microangiopathy evolution score; mRSS: modified Rodnan Skin Score; NT-proBNP: N-terminal prohormone of brain natriuretic peptide; PAH: pulmonary arterial hypertension; RV: residual volume; RV/LV: right ventricle to left ventricle; TAPSE: tricuspid annular plane systolic excursion; TLC: total lung capacity.

Table II. Patients' characteristics at study inclusion.

Patient characteristics	LcSSc	Controls	*p*-value
Number of patients, n (%)	38 (50)	38 (50)	>0.999
Sex, n (%)			
Male	2 (5.3)	2 (5.3)	>0.999
Female	36 (94.7)	36 (94.7)	>0.999
Age (years), mean (± SD)	57.9 ± 9.2	57.2 ± 9.0	0.622
Body mass index (kg/m^2), mean (± SD)	23.61 ± 3.46	22.86 ± 3.53	0.487
Smoking, n (%)			
Active smokers	4 (10.5)	7 (18.4)	0.516
Ex-smokers	8 (21.1)	8 (21.1)	>0.999
Arterial hypertension, n (%)	14 (36.8)	12 (31.6)	0.809
Hyperlipidemia, n (%)	20 (52.6)	12 (31.6)	0.103
Diabetes mellitus, n (%)	0 (0)	0 (0)	-
HbA1c (mmol/mol), median (25-75th percentile)	35 (34-37)	37 (34-38)	0.308
Prior atherosclerotic disease, n (%)			
Coronary artery disease	0 (0)	0 (0)	-
Insult or transient ischaemic attack	0 (0)	0 (0)	-
Peripheral artery disease	0 (0)	0 (0)	-
Malignancies, n (%)			
Recent	0 (0)	0 (0)	-
Prior	4 (10.5)	1 (2.6)	0.358
Prior digital ulcers, n (%)	0 (0)	0 (0)	-
Prior PAH, n (%)	0 (0)	0 (0)	-
Prior interstitial lung disease, n (%)	2 (5.3)	0 (0)	0.493
Prior oesophageal involvement, n (%)	9 (23.7)	0 (0)	**0.002**
Prior renal involvement, n (%)	2 (5.3)	0 (0)	0.493
Prior xerostomia/xerophthalmia, n (%)	23 (60.5)	0 (0)	**<0.001**
Prior primary biliary cholangitis, n (%)	3 (7.9)	0 (0)	0.240
Medication, n (%)			
ACE inhibitors/ARB	7 (18.4)	6 (15.8)	>0.999
Beta blockers	3 (7.9)	4 (10.5)	>0.999
Calcium channel blockers	6 (15.8)	5 (13.2)	>0.999
Diuretics	2 (5.3)	3 (7.9)	>0.999
Platelet aggregation inhibitors	6 (15.8)	4 (10.5)	0.736
Anticoagulants	3 (7.9)	0 (0)	0.240
Statins	3 (7.9)	3 (7.9)	>0.999
Immunosuppression, n (%)	6 (15.8)	0 (0)	**0.025**
Cortisone	3 (7.9)	0 (0)	0.240
Methotrexate	1 (2.6)	0 (0)	>0.999
Mycophenolate mofetil	2 (5.3)	0 (0)	0.493
Rituximab	1 (2.6)	0 (0)	>0.999
Hydroxychloroquine	2 (5.3)	0 (0)	0.493
Abatacept	1 (2.6)	0 (0)	>0.999

ACE: angiotensin converting enzyme; ARB: Angiotensin II receptor blockers; PAH: pulmonary arterial hypertension.

sequently, NVC (Skinview, Optometron Ltd., Oskar-Messterstr., Ismaning, Germany) of the second to fifth digit on both hands was performed in sitting position and one 1-mm-sized image per digit was captured and stored. Morphological changes of the capillaries were recorded and semiquantitative rating scale to score each capillary abnormality was adopted (0 = no changes; 1 = less than 33% of capillary changes; 2 = 33–66% of capillary changes; 3 = more than 66% of capillary changes, per linear millimetre). The score values from the eight digits were added together and divided by eight resulting in the final score values. Microvascular disease activity was assessed by capillaroscopic skin ulcer risk index (CSURI) and microangiopathy evolution score (MES) (32, 33). Spirometry and DLCO were performed according to recent guidelines (34, 35).

Statistical analysis

Continuous variables were given as means and standard deviation (SD) or median and interquartile range and categorical variables were represented by frequency and percentages. The normal distribution was examined with Kolmogorov-Smirnov test. Student's *t*-test was used for normally distributed data and Mann-Whitney U-test was used for non-normally distributed data. Categorical variables were analysed by Chi-square test. Spearman's and Pearson's correlation coefficient were used for non-normally and normally distributed variables respectively. Statistical significance was assumed for *p*-values<0.05 and statistical analyses were executed via SPSS v. 26.0.

Ethical approval

The study was approved by the Institutional Review Board of the Medical University Graz, Austria (EK 29-361 ex 16/17). All patients gave their written informed consent after being accurately informed about that clinical trial.

Results

89 patients with lcSSc were screened for study participation. Of those, 28 patients were excluded due to ≥1 exclusion criterion, 22 patients declined study participation and 1 patient died before study visit was performed. Finally, 38 Caucasian patients with lcSSc (36 female, 94.7%) and 38 age-, race- and sex-matched controls with primary Raynaud's phenomenon participated in that study. Patient characteristics are shown in Table II.

Endothelial dysfunction

FMD did not differ between patients with lcSSc and controls (4.53±3.67% *vs*. 4.75±2.99%, *p*=0.775). Additionally, neither NMD, nor PWV, nor Aix revealed significant differences between both groups. NMD was not performed in 10 subjects due to a low blood pressure (<100/70mmHg) at study measurement. According to the reported reference values for FMD, NMD and PWV, 31 patients with lcSSc and 28 controls had a FMD <7% (81.6% *vs*. 73.7%, *p*=0.583), 7 lcSSc patients and 6 controls had a NMD <15.6% (21.2% *vs*. 18.2%, *p*>0.999), and 5 lcSSc patients and 8 controls had a PWV >10m/s (13.2% *vs*. 21.1%, *p*=0.544). Patients with lcSSc had higher values of ADMA and SDMA, while the remaining parameters of the arginine

Table III. Bivariate analysis of parameters of endothelial dysfunction.

	LcSSc	Controls	*p*-value
FMD (%), mean (± SD)	4.53 ± 3.67	4.75 ± 2.99	0.775
<7%, n (%)	31 (81.6)	28 (73.7)	0.583
NMD (%), mean (± SD)	20.78 ± 9.09	22.02 ± 7.88	0.303
<15.6%, n (%)	7 (21.2)	6 (18.2)	>0.999
Aortic PWV (m/s), mean (± SD)	8.26 ± 1.52	8.49 ± 1.67	0.662
>10m/s, n (%)	5 (13.2)	8 (21.1)	0.544
Aix, mean (± SD)	26.55 ± 13.12	28.08 ± 12.15	0.600
ADMA (µmol/L), median (25-75th percentile)	0.66 (0.58-0.73)	0.63 (0.57-0.67)	**0.030**
SDMA (µmol/L), median (25-75th percentile)	0.65 (0.57-0.76)	0.62 (0.54-0.70)	**0.025**
Arginine (µmol/L), median (25-75th percentile)	111.9 (102.2-122.2)	111.3 (100.6-125.4)	0.580
Homoarginine (µmol/L), median (25-75th percentile)	1.64 (1.17-2.08)	1.70 (1.25-2.11)	0.633
Citrulline (µmol/L), median (25-75th percentile)	33.65 (30.44-38.31)	34.99 (29.14-39.14)	0.857
Ornithine (µmol/L), median (25-75th percentile)	74.36 (66.19-84.35)	68.28 (62.25-82.98)	0.377
Arginine/ADMA ratio, median (25-75th percentile)	174.5 (155.6-190.7)	182.3 (157.2-207.3)	0.312
Arginine/SDMA ratio, median (25-75th percentile)	168.2 (148.2-201.1)	182.5 (159.9-222.4)	0.189
Homoarginine/ADMA ratio, median (25-75th percentile)	2.40 (1.93-3.17)	2.66 (1.94-3.85)	0.340
Homoarginine/SDMA ratio, median (25-75th percentile)	2.37 (1.76-3.12)	2.70 (1.78-3.89)	0.194
Arginine/citrulline ratio, median (25-75th percentile)	3.27 (3.00-3.68)	3.19 (2.79-4.13)	0.838
GAB ratio, median (25-75th percentile)	1.05 (0.92-1.18)	1.11 (0.94-1.24)	0.831
EMP (U/µl), median (25-75th percentile)			
CD31+/CD42b-	29 (22-40)	24 (16-31)	0.062
CD51+/CD42b-	n.d.	n.d.	-
CD54+/CD42b-	n.d.	n.d.	-
CD62E+/CD42b-	n.d.	n.d.	-
CD105+/CD42b-	n.d.	n.d.	-
CD144+/CD42b-	n.d.	n.d.	-

ADMA: asymmetric dimethylarginine; Aix: augmentation index; EMP: endothelial-derived microparticles; FMD: flow-mediated dilation; GAB: global arginine bioavailability; NMD: nitroglycerine-mediated dilation; n.d.: not detectable; PWV: pulse-wave velocity; SDMA: symmetric dimethylarginine.

metabolism did not significantly differ between both groups (Table III). There was borderline significance for CD31+/CD42b- EMP, which were also included in further statistical analysis, while CD51+/CD42b-, CD54+/CD42b-, CD62E+/CD42b-, CD105+/CD42b-, and CD144+/CD42b- EMP were undetectable in both groups (Table III).

Significantly positive correlations were found between ADMA and SDMA, ADMA and CD31+/CD42b- EMP, and ADMA and citrulline. Citrulline correlated also positively with arginine. Aix revealed a positive correlation with CD31+/CD42b- EMP and PWV, while negative correlation between FMD and PWV was found. Additional positive correlation was observed between FMD and NMD. No further significant correlations were found between parameters of the arginine metabolism, arterial stiffness, vascular reactivity or CD31+/CD42b- EMP (Table IV). No correlation analysis was conducted for the ratios of the arginine metabolism as those ratios represent numerical proportions of the respective parameters.

Clinical events

Clinical events were compared in bivariate analysis between lcSSc patients and controls. Patients with lcSSc had significantly more potential signs of PAH and a higher DETECT score than controls while parameters for potential lung fibrosis did not differ between both groups. Higher values of creatinine and lower values of estimated glomerular filtration rate (eGFR) were found in lcSSc patients while no difference for the remaining renal parameters were observed. The number of cases comparing urine immunoglobulin G and β2-microglobulin/creatinine ratio between both groups was too low to achieve adequate statistical analysis. Patients with lcSSc had more frequently capillaroscopic changes, including giant capillaries, microhaemorrhages, capillary ramifications, bushy capillaries, capillary loss, capillary oedema and disorganisation of microvascular array. Higher semiquantitative values of the respective capillary changes, a higher overall MES and a higher CSURI were also observed in lcSSc patients. Additionally, active and late pattern were more frequently present in lcSSc patients, while early pattern achieved borderline significance. There was no difference for impaired acral perfusion between both groups. Higher values of mRSS were observed in patients with lcSSc, which have also more frequently clinical skin changes, including telangiectasia, sclerodactyly and puffy fingers. Neither total score of the UCLA SCTC GIT 2.0 questionnaire nor feeling of xerophthalmia or xerostomia differed between patients with lcSSc and controls. EUSTAR index were higher in patients with lcSSc than in controls, although an EUSTAR index ≥2.5 indicating active disease was observed in only 2 patients (5.3%) with lcSSc (Table V).

In patients with lcSSc, SDMA correlated significantly with the number of points of step 1 and step 2 DETECT score and ADMA revealed also positive correlation with the points of step 2 DETECT score while borderline correlation was achieved for the points of step 1 DETECT score. ADMA and CD31+/CD42b- EMP correlated positively with CSURI and SDMA correlated negatively with eGFR. No further correlations were observed between parameters of endothelial dysfunction and clinical events (Table VI).

Discussion

As previous data reported that vascular changes are more common in lcSSc and that different parameters of endothelial

Table IV. Correlation matrix of parameters of endothelial dysfunction.

		ADMA	SDMA	Arginine	Homoarginine	Citrulline	Ornithine	CD31+/ CD42b- EMP	Aix	PWV	FMD
SDMA	r	.649									
	p	.000									
	n	76									
Arginine	r	.143	.070								
	p	.217	.549								
	n	76	76								
Homoarginine	r	-.019	-.131	.117							
	p	.872	.261	.312							
	n	76	76	76							
Citrulline	r	.262	.219	.292	.026						
	p	.022	.057	.011	.826						
	n	76	76	76	76						
Ornithine	r	.184	.070	.080	-.040	.189					
	p	.112	.545	.490	.730	.102					
	n	76	76	76	76	76					
CD31+/CD42b- EMP	r	.236	.217	-.133	.107	.063	.194				
	p	.040	.060	.251	.358	.588	.094				
	n	76	76	76	76	76	76				
Aix	r	-.097	-.016	-.179	.022	-.126	.037	.252			
	p	.405	.890	.121	.849	.277	.751	.028			
	n	76	76	76	76	76	76	76			
PWV	r	.027	.066	-.014	-.221	.092	.058	.132	.345		
	p	.815	.570	.903	.055	.430	.620	.255	.002		
	n	76	76	76	76	76	76	76	76		
FMD	r	.089	.000	-.134	.195	-.038	.020	.171	-.022	-.411	
	p	.443	.999	.248	.091	.743	.863	.140	.847	.000	
	n	76	76	76	76	76	76	76	76	76	
NMD	r	-.085	-.212	.071	.113	-.229	.113	.034	.091	-.210	.541
	p	.498	.088	.570	.368	.065	.367	.784	.469	.091	.000
	n	66	66	66	66	66	66	66	66	66	66

ADMA: asymmetric dimethylarginine; Aix: augmentation index; EMP: endothelial-derived microparticles; FMD: flow-mediated dilation; NMD: nitroglycerine-mediated dilation; PWV: pulse-wave velocity; SDMA: symmetric dimethylarginine.

dysfunction are significantly changed in lcSSc compared to dcSSc, this study aimed to use lcSSc patients without end-stage vasculopathy (17, 36-38). While the underlying mechanisms contributing to vasculopathic changes between different SSc subtypes need to be elucidated, this study demonstrated that selected parameters of endothelial dysfunction are altered and contribute to clinical changes in lcSSc patients already at early-stage vasculopathy. Although neither FMD nor NMD differed between both groups, values of FMD and NMD were similarly decreased compared to previous studies (3, 5, 18). Furthermore, 81.6% of lcSSc patients had reduced FMD values while 21.2% had reduced NMD values compared to proposed references suggesting that endothelial-dependent vasodilation reflected by FMD is largely impaired in lcSSc. Contrary, only 13.2% of lcSSc patients had an aortic PWV >10% suggesting that arterial compliance is largely prevailed in lcSSc. Although Meiszterics *et al*. (6) reported in a meta-analysis decreased FMD and increased PWV values in SSc, individual studies reported varying data. That variation is primarily caused due to heterogenous investigational methods, including missing controls, absent age- or sex-matching, inclusion of different SSc subtypes as well as inclusion of end-stage vasculopathic manifestations of SSc, like PAH or digital ulcers, or concomitant cardiovascular risk factors (3, 5, 18-22). All might influence results of vascular reactivity and arterial stiffness. Furthermore, positive correlations between FMD and NMD as well as between PWV and Aix were observed, which was however expected as these variables describe the same characteristics. An additional negative correlation between FMD and PWV suggests that impaired vascular reactivity and increased arterial stiffness are promoted by the same pathologic processes of the arterial wall. Moreover, Aix correlated

Table V. Bivariate analysis of clinical events between patients with lcSSc and controls.

Clinical event and disease-specific parameter	LcSSc	Controls	*p*-value
PAH			
Potential signs of PAH (n), mean (± SD)	2 ± 1	1 ± 1	**0.020**
DETECT score (points), mean (± SD)			
DETECT score step 1	309 ± 16	290 ± 12	**<0.001**
DETECT score step 2	29 ± 6	22 ± 4	**<0.001**
Signs of lung fibrosis, mean (± SD)			
Predicted FVC%	106 ± 18	104 ± 15	0.408
Predicted FEV1%FVC	78 ± 6	76 ± 7	0.240
Predicted RV%	117 ± 19	118 ± 27	0.862
Predicted TLC%	111 ± 13	113 ± 11	0.526
Predicted single breath DLCO%	89 ± 15	97 ± 17	0.073
Gastrointestinal involvement			
UCLA SCTC GIT 2.0 (points), mean (± SD)	0.30 ± 0.37	0.23 ± 0.24	0.639
Sicca symptoms			
Feeling of xerostomia/xerophthalmia, n (%)	24 (63.2)	22 (57.9)	0.815
Skin involvement			
mRSS (points), mean (± SD)	4.84 ± 3.84	0.47 ± 0.95	**<0.001**
Telangiectasia, n (%)	11 (28.9)	0 (0)	**<0.001**
Calcinosis cutis, n (%)	1 (2.6)	0 (0)	>0.999
Puffy finger, n (%)	15 (39.5)	0 (0)	**<0.001**
Sclerodactyly, n (%)	22 (57.9)	0 (0)	**<0.001**
Acral necrosis, n (%)	0 (0)	0 (0)	-
Tendon friction rub, n (%)	0 (0)	0 (0)	-
Renal involvement, median (25-75th percentile)			
eGFR (mL/min/1.73m^2)	78.42 (71.60-90.86)	88.85 (76.74-95.76)	**0.008**
Creatinine (mg/dL)	0.85 (0.74-0.96)	0.75 (0.69-0.86)	**0.042**
Protein/creatinine ratio (mg/g of creatinine)	95 (82-110)	106 (75-133)	0.229
Urine immunoglobulin G (mg/L)	-*	-*	-
Albumin/creatinine ratio (mg/g of creatinine)	13 (7-18)	15 (7-21)	0.746
α1-microglobulin/creatinine ratio (mg/g of creatinine)	11 (8-13)	11 (5-13)	0.950
β2-microglobulin/creatinine ratio (mg/g of creatinine)	-*	-*	-
Microvascular involvement			
Impaired acral perfusion, n (%)	37 (97.4)	36 (94.7)	>0.999
Early pattern, n (%)	5 (13.2)	0 (0)	0.054
Active pattern, n (%)	9 (23.7)	0 (0)	**0.002**
Late pattern, n (%)	11 (28.9)	0 (0)	**<0.001**
Giant capillaries, n (%)	14 (36.8)	0 (0)	**<0.001**
Giant capillaries (points), mean (± SD)	0.22 ± 0.43	0.00 ± 0.00	**<0.001**
Microhaemorrhages, n (%)	23 (60.5)	3 (7.9)	**<0.001**
Microhaemorrhages (points), mean (± SD)	0.25 ± 0.32	0.01 ± 0.03	**<0.001**
Capillary ramifications, n (%)	14 (36.8)	0 (0)	**<0.001**
Capillary ramifications (points), mean (± SD)	0.16 ± 0.29	0.00 ± 0.00	**<0.001**
Capillary loss, n (%)	9 (23.7)	0 (0)	**0.002**
Capillary loss (points), mean (± SD)	0.12 ± 0.33	0.00 ± 0.00	**0.002**
Capillary oedema, n (%)	8 (21.1)	0 (0)	**0.005**
Capillary oedema (points), mean (± SD)	0.24 ± 0.56	0.00 ± 0.00	**0.005**
Disorganisation of microvascular array, n (%)	37 (97.4)	24 (63.2)	**<0.001**
Disorganisation of microvascular array (points), mean (± SD)	2.12 ± 0.92	0.76 ± 0.70	**<0.001**
Bushy capillaries, n (%)	14 (36.8)	5 (13.2)	**0.032**
Bushy capillaries (points), median (25-75th percentile)	0.13 ± 0.23	0.03 ± 0.09	**0.008**
CSURI (points), median (25-75th percentile)	4.85 ± 12.22	-§	**<0.001**
MES (points), median (25-75th percentile)	2.41 ± 1.12	0.76 ± 0.70	**<0.001**
EUSTAR index (points), mean (± SD)	0.66 ± 0.73	0.09 ± 0.23	**<0.001**
EUSTAR index ≥ 2.5, n (%)	2 (5.3)	0 (0)	0.493

CSURI: capillaroscopic skin ulcer risk index; DLCO: diffusing capacity; EUSTAR: European Scleroderma Trials and Research Group; FEV1: forced expiratory volume in 1 second; FVC: forced vital capacity; eGFR: estimated glomerular filtration rate; MES: microangiopathy evolution score; mRSS: modified Rodnan Skin Score; PAH: pulmonary arterial hypertension; RV: residual volume; TLC: total lung capacity.

*The number of cases between both groups was too low to achieve adequate statistical analysis.

§CSURI score is not applicable as none of the controls had giant capillaries.

with CD31+/CD42b- EMP, but not with PWV, indicating that CD31+/CD42b- EMP may contribute to arterial stiffness only to a certain degree. However, as no parameter of the arginine metabolism correlated with FMD, NMD, PWV or Aix, further underlying mechanisms may contribute to a reduced NO synthesis and arterial compliance in lcSSc.

Parameters of the arginine metabolism are important mediators of the NO metabolism and predictors of cardiovascular diseases, while their contribution in SSc-related endothelial dysfunction is less investigated. So far, elevated levels of ADMA have been reported mainly in SSc patients with end-stage vasculopathic manifestations including PAH or digital ulcers while data about ADMA in early-stage vasculopathy and data about the remaining parameters of the arginine metabolism in SSc are limited (15, 18, 20). In this study, ADMA and SDMA were elevated in patients with lcSSc, suggesting that both may promote to endothelial dysfunction in lcSSc already at early-stage vasculopathy. However, and contrary to atherosclerotic cardiovascular disease, there was no difference between both groups for the remaining parameters (39). ADMA revealed a predictably positive correlation to SDMA and to citrulline as both, ADMA and SDMA, are released and formed via proteolysis by protein arginine methyltransferases and ADMA is catabolised by dimethyl-diamino-arginine-hydrolase enzymes to citrulline. Furthermore, arginine and citrulline correlated also with each other as citrulline can be metabolised to arginine (11). Reducing increased ADMA or SDMA levels may be a reasonable goal for the treatment of endothelial dysfunction in lcSSc. However therapeutic options are limited, including pravastatin, metformin or supplementation of L-arginine, and the effects on endothelial dysfunction were mostly not investigated in SSc or failed to achieve statistical significance (40, 41). Further studies are needed to clarify the influence of different drugs on endothelial dysfunction in lcSSc.

EMP are derived from endothelial activation or apoptosis and there are numerous EMP phenotypes identified by

Table VI. Correlation matrix of selected parameters of endothelial dysfunction and selected clinical events within lcSSc patients.

		ADMA	SDMA	CD31+/ CD42b- EMP
mRSS	r	-.103	-.214	-.211
	p	.539	.198	.204
	n	38	38	38
DETECT score step 1	r	.307	.376	.098
	p	.061	.020	.560
	n	38	38	38
DETECT score step 2	r	.328	.426	.178
	p	.045	.008	.285
	n	38	38	38
eGFR	r	-.233	-.560	.040
	p	.159	.000	.813
	n	38	38	38
MES	r	-.132	-.083	-.061
	p	.431	.619	.715
	n	38	38	38
CSURI	r	.329	.188	.327
	p	.043	.258	.045
	n	38	38	38
Early pattern	r	-.210	-.018	-.078
	p	.213	.925	.625
	n	38	38	38
Active pattern	r	-.234	-.178	-.240
	p	.159	.288	.149
	n	38	38	38
Late pattern	r	.249	.106	.214
	p	.134	.530	.198
	n	38	38	38
EUSTAR index	r	.062	-.177	-.216
	p	.710	.288	.193
	n	38	38	38

ADMA: asymmetric dimethylarginine; CSURI: capillaroscopic skin ulcer risk index; eGFR: estimated glomerular filtration rate; EMP: endothelial-derived microparticles; EUSTAR: European Scleroderma Trials and Research Group; SDMA: symmetric dimethylarginine; MES: microangiopathy evolution score; mRSS: modified Rodnan Skin Score.

specific cell-surface markers contributing to endothelial inflammation, structural endothelial modification and angiogenesis (13, 42). Interestingly, only CD31+/CD42b- EMP were detected in this study while other EMP phenotypes were undetectable, contrary to previous reports. One reason may be technical aspects as we used silica particles for size calibration instead of polystyrene particles and fluorescence triggering on lactadherin fluorescence for the detection of EMP, which differed from previous studies (16,17,43). Additionally, EMP can be falsified by various influencing factors, including venous stasis, shaking or haemolysis of the blood sample (44). Although venous stasis and shaking were avoided and slight haemolysis was present in only 1 sample, further unknown factors might have influenced the samples. Another reason may be that other EMP phenotypes than CD31+/CD42b- EMP were present in lcSSc patients only at end-stage vasculopathy. Additional positive correlation of CD31+/CD42b- EMP and ADMA and also borderline significant correlation with SDMA were observed suggesting potential interaction between EMP release and dimethylarginines. So far, potential inhibition of NO production by elevated EMP was reported in mouse hearts, but data about interaction of EMP and arginine metabolism are yet lacking (45).

Significant differences of clinical events were observed for SSc-related skin changes including mRSS, capillaroscopic changes and scores, and EUSTAR index, which was however expectable as those parameters are SSc-specific. Although PAH was an exclusion criterion and only 2 patients had prior SSc-related renal involvement, higher DETECT prediction score, more clinical parameters of potential PAH, and higher levels of creatinine and lower levels of eGFR were observed in lcSSc patients suggesting that changes contributing to subclinical PAH and nephropathy may be already present in those patients with early-stage vasculopathy. Our results suggest that subclinical PAH may be promoted by ADMA and SDMA as both correlated with the DETECT score. Additionally, ADMA and CD31+/CD42b- EMP may contribute to the development of digital ulcers in naïve lcSSc patients without digital ulcers as both correlated with CSURI. CD31+/CD42b- EMP showed an inverse correlation with CSURI suggesting that CD31+/CD42b- EMP reflect rather the degree of endothelial apoptosis than endothelial activation, as also a positive but not significant correlation to capillary late pattern was observed. These findings are contrary to previous results in which negative correlations to capillary changes of late pattern were found (16). Further studies are needed to clarify the contribution of EMP with capillary changes in lcSSc.

No differences were found for signs of lung fibrosis, gastrointestinal involvement and sicca symptoms between both groups which may be attributed to insufficiently investigational methods as neither computed tomography of the lungs nor objective measurements of gastrointestinal involvement and sicca symptoms were performed.

Limitations of this study are a relatively small sample size and lacking objective measurements for selected clinical events, while the strengths are a homogenous cohort of lcSSc patients defined by the recent ACR/EULAR criteria without disease manifestations indicating end-stage vasculopathy and inclusion of age-, race- and sex-matched controls with comparable comorbidities. Furthermore, it can be presumed that the usage of patients with primary Raynaud's phenomenon was adequate as these patients did not exhibit pathologic values of FMD and NMD (46).

In conclusion, selected parameters of endothelial dysfunction contribute to microvascular, clinical changes in lcSSc patients with early-stage vasculopathy while their impact on vascular reactivity and arterial stiffness remains still indistinctively.

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