Dissertation

Endothelial dysfunction in patients with limited cutaneous systemic sclerosis

submitted by

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2021

1. Statutory declaration

I hereby declare that this thesis is my own original work and that I have fully acknowledged by name all of those individuals and organizations that have contributed to the research for this thesis. Due acknowledgement has been made in the text to all other material used. Throughout this thesis and in all related publications I followed the “Standards of Good Scientific Practice and Ombuds Committee at the Medical University of Graz”.

Graz, 01.07.2021

2. Disclosures

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All of the contributing authors have explicitly agreed to the use of their data in this thesis.

Permission to reproduce figures and tables have been obtained from the journal. The author of the dissertation hereby confirms that he has permission to reproduce the illustrations and tables as well as text passages used in this work. If necessary, these were provided with the corresponding references.

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5. List of abbreviations

ACA: Anti-centromere autoantibodies

ACE: Angiotensin-converting-enzyme

ACR: American College of Rheumatology

ADMA: Asymmetric dimethylarginine

AECA: Anti-endothelial-cell antibodies

AIx: Augmentation index

ANA: Antinuclear autoantibodies

Anti-CENP-B: Antibodies against centromere protein B

Anti-PM-Scl: Antibodies against PM-Scl-100 and PM-Scl-75 proteins

Anti-RNAP: Antibodies against RNA polymerase

Anti-Th/To: Antibodies against H1/8-2 and Th/7-2 RNA

Anti-U1-RNP: Antibodies against 70 kDa A and C polypeptides of U1 snRNP

aPTT: Activated partial thromboplastin time

aPWV: Aortic pulse wave velocity

ARB: Angiotensin II receptor blocker

ATA: Antitopoisomerase I autoantibodies

BHPR: British Health Professionals in Rheumatology

BOP: Bleeding on probe

BSR: British Society for Rheumatology

CRP: C-reactive protein

CSURI: Capillaroscopic skin ulcer risk index

DcSSc: Diffuse cutaneous systemic sclerosis

DLCO: Diffusing capacity of the lung for carbon monoxide

DNA: Deoxyribonucleic acid

ECG: Electrocardiogram

eGFR: estimated glomerular filtration rate

EMP: Endothelial-derived microparticles

ENA: Extractable nuclear antigens

eNOS: Endothelial nitric oxide synthase

ESR: Erythrocyte sedimentation rate

EULAR: European league against rheumatism

FMD: Flow-mediated dilation

HDL: High-density lipoprotein

HLA: Human leucocyte antigen

ICAM-1: Intercellular adhesion molecule-1

IFN: Interferon

IQR: interquartile range

IL: Interleukin

LcSSc: Limited cutaneous systemic sclerosis

LDL: Low-density lipoprotein

MCP: Monocyte chemotactic protein

mRSS: Modified Rodnan Skin Score

NMD: Nitroglycerine-mediated dilation

NO: Nitric oxide

NT-proBNP: N-terminal prohormone of brain natriuretic peptide

NVC: Nailfold videocapillaroscopy

PAH: Pulmonary arterial hypertension

PDGF: Platelet-derived growth factor

PWV: Pulse wave velocity

RV/LV: right-ventricle-to-left-ventricle

RNA: Ribonucleic acid

SD: standard deviation

SDMA: Symmetric dimethylarginine

SMAD: Small mothers against decapentaplegic homologues

SSc: Systemic sclerosis

T3: Triiodothyronine

T4: Thyroxine

TAPSE: Tricuspid annular plane systolic excursion

TGF: Transforming growth factor

TNF: Tumor necrosis factor

TSH: Thyroid-stimulating hormone

VCAM-1: Vascular cell adhesion molecule-1

VEGF: Vascular endothelial growth factor

vWF: von Willebrand factor

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8. Zusammenfassung

Hintergrund:

Die limitierte kutane systemische Sklerose (lcSSc) ist eine autoimmune Bindegewebserkrankung, die durch eine Vaskulopathie, Autoimmunaktivierung und Bindegewebsfibrose charakterisiert ist. Die Vaskulopathie wird durch die Endotheldysfunktion begünstigt, was folglich zu einem Gefäßschaden mit Apoptose der Endothelzellen und zu strukturellen Veränderungen der Mikrozirkulation führt. Bislang sind nur wenige Daten zur Endothelfunktion bei Patienten mit lcSSc vorhanden. Ziele dieser Studie waren Parameter der Endotheldysfunktion, inklusive Fluss-mediierter Dilatation (FMD), Nitroglyzerin-mediierter Dilatation (NMD), aortaler Pulswellengeschwindigkeit (aPWV), endothelialer Mikropartikel (EMP), Arginin, Homoarginin, asymmetrischen Dimethylarginin (ADMA) und symmetrischen Dimethylarginin (SDMA) zu untersuchen, sowie die Korrelationen dieser Parameter untereinander, mit Entzündungsparametern, und mit klinischen Parametern, darunter vaskuläre Ereignisse und Parodontitis, bei Patienten mit lcSSc zu erforschen. Zusätzlich wurden mögliche Unterschiede dieser Parameter innerhalb der Patientengruppe mit lcSSc zwischen einer Subgruppe mit einer Krankheitsdauer von ≤ 7 Jahren zu einer Subgruppe mit einer Krankheitsdauer von > 7 Jahren untersucht.

Methoden:

Patienten mit bekannter lcSSc sowie alters-, ethnien-, und geschlechtsentsprechende Kontrollen mit einem primären Raynaud-Phänomen wurden auf eine mögliche Studienteilnahme überprüft. Bei allen Patienten mit einer lcSSc und bei allen Kontrollen wurde die FMD, NMD sowie aPWV gemessen und es erfolgte eine biochemische Untersuchung von Entzündungswerten, EMP, Arginin, Homoarginin, ADMA und SDMA. Vaskuläre Ereignisse wurde als mögliche Zeichen eines vorhandenen pulmonal-arteriellen Hypertonus, Sicca-Symptome, dermale, mikrovaskuläre, renale und gastrointestinale Beteiligung definiert und durch Anamnese inklusive eines spezifischen Fragebogens, physikalischer Untersuchung, Elektrokardiogramm, transthorakalem Rechtsherz-Ultraschall, digital-akrale Pulsoszillografie, Kapillarmikroskopie, Spirometrie sowie Laboruntersuchung erhoben. Zusätzlich erfolgte die Durchführung einer zahnärztlichen Untersuchung zur Evaluierung parodontitischer Veränderungen.

Ergebnisse:

Nach Überprüfung auf eine Studienteilnahme wurden 38 Patienten mit lcSSc und einem Durchschnittsalter (± Standardabweichung) von 57,89 ± 9,22 Jahren sowie einer durchschnittlichen Krankheitsdauer (± Standardabweichung) von 7,11 ± 5,78 Jahren und 38 alters-, ethnien-, und geschlechtsentsprechende Kontrollen mit einem Durchschnittsalter (± Standardabweichung) von 57,20 ± 8,96 Jahren und einer durchschnittlichen Krankheitsdauer (± Standardabweichung) von 5,70 ± 3,17 Jahren eingeschlossen. Weder FMD (p=0,775), noch NMD (p=0,303), noch aPWV (p=0,662) unterschieden sich zwischen Patienten mit lcSSc und Kontrollen. Bei Patienten mit lcSSc wurden höhere Werte für ADMA (p=0,030) und SDMA (p=0,025) gefunden, während Arginin (p=0,580), Homoarginin (p=0,663) und CD31+/CD42b- EMP (p=0,062) keinen Unterschied zwischen Patienten mit lcSSc und Kontrollen zeigten. Es wurden signifikante Korrelationen zwischen FMD und NMD, FMD und aPWV, ADMA und SDMA sowie ADMA und CD31+/CD42b-EMP gefunden (p<0,05 jeweils). Signifikante Unterschiede bestanden auch zwischen Patienten mit lcSSc und Kontrollen hinsichtlich bestimmter Haut- und Kapillarveränderungen, dem DETECT-Score, Kreatinin und der geschätzten glomerulären Filtrationsrate (p<0,05 jeweils).

Zwischen Patienten mit einer lcSSc und einer Krankheitsdauer von ≤ 7 Jahren sowie Patienten mit lcSSc und einer Krankheitsdauer von > 7 Jahren konnte eine höhere Rate von Sklerodaktylien (p=0,020) in der lcSSc-Subgruppe mit einer Krankheitsdauer von > 7 Jahren beobachtet werden, wohingegen sich alle anderen Parameter der Endothelfunktion, vaskulären Ereignisse und Parodontitis nicht unterschieden.

Konklusion:

Die Endotheldysfunktion betrifft bei Patienten mit lcSSc hauptsächlich die Mikrozirkulation, während ihre Auswirkung auf das makrovaskuläre System unklar bleibt. Positive Korrelationen zwischen ADMA, SDMA und CD31+/CD42b- EMP deuten auf eine mögliche Wechselwirkung zwischen Endothelaktivierung und NO-Metabolismus bei Patienten mit lcSSc hin.

9. Abstract

Background:

Limited cutaneous systemic sclerosis (lcSSc) is an autoimmunological, connective tissue disorder characterized by vasculopathy, autoimmune activation and tissue fibrosis. Vasculopathy is promoted by endothelial dysfunction contributing to vascular damage including endothelial apoptosis and structural changes of the microvasculature. Only limited data are yet available for endothelial dysfunction in patients with lcSSc. The aims of this study were to investigate endothelial dysfunction, assessed by flow-mediated dilation (FMD), nitroglycerine-mediated dilation (NMD), aortic pulse-wave velocity (aPWV), endothelial microparticles (EMP), arginine, homoarginine, asymmetric dimethylarginine (ADMA), and symmetric dimethylarginine (SDMA), and the correlations of those parameters between each other, with inflammatory parameters, as well as with clinical parameters, including vascular events and periodontal inflammation, in patients with lcSSc. Furthermore, potential differences of those parameters were also investigated within the group of patients with lcSSc between those with a disease duration of ≤ 7 years and those with a duration > 7 years.

Methods:

Patients with known lcSSc as well as age-, race- and sex-matched patients with primary Raynaud's phenomenon using as controls were screened for eligibility. All patients with lcSSc and controls underwent measurements of FMD, NMD, aPWV, and biochemical analysis including inflammatory parameters, EMP, arginine, homoarginine, ADMA, and SDMA. Vascular events defined as signs of pulmonary arterial hypertension, sicca symptoms, skin, microvascular, renal, and gastrointestinal involvement were recorded by medical history including a disease-specific questionnaire, physical examination, electrocardiogram, transthoracic right heart echocardiogram, digital acral plethysmography, nailfold videocapillaroscopy, spirometry, and laboratory parameters. Additionally, dental and oral examination was performed for evaluation of periodontal inflammation.

Results:

After screening for eligibility, 38 patients with lcSSc with a mean age (± standard deviation [SD]) of 57.89 ± 9.22 years and a mean disease duration (± SD) of 7.11 ± 5.78 years as well as 38 age-, race- and sex-matched controls with a mean age (± SD) of 57.20 ± 8.96 years and a mean disease duration (± SD) of 5.70 ± 3.17 years were included. Neither FMD (p=0.775) nor NMD (p=0.303) nor aPWV (p=0.662) differed between patients with lcSSc and controls. In

patients with lcSSc, higher values of ADMA (p=0.030) and SDMA (p=0.025) were observed, while arginine (p=0.580), homoarginine (p=0.663) and CD31+/CD42b- EMP (p=0.062) did not differ between patients with lcSSc and controls. Significant correlations were found between FMD and NMD, FMD and aPWV, ADMA and SDMA, and ADMA and CD31+/CD42b- EMP (all $p<0.05$). Significant differences between patients with lcSSc and controls were observed for selected skin and capillary changes, DETECT score, creatinine and estimated glomerular filtration rate ($p<0.05$).

Between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years, only a higher rate of sclerodactyly (p=0.020) was observed in lcSSc patients with a disease duration of > 7 years, while all other parameters of endothelial dysfunction, vascular events and periodontal inflammation did not differ.

Conclusion:

Endothelial dysfunction affects mainly the microvascular system in patients with lcSSc while its impact on the macrovascular system remains indistinctively. Positive correlations between ADMA, SDMA and CD31+/CD42b- EMP suggest potential interaction of endothelial activation and NO metabolism in patients with lcSSc.

10. Introduction

10.1 Background of systemic sclerosis

10.1.1 Definition and classification

Scleroderma is an autoimmune disease and can be classified as a connective tissue disorder which is characterized by cutaneous and visceral fibrosis. Scleroderma can be divided into systemic sclerosis (SSc) and localized scleroderma. While localized scleroderma affecting only the skin, SSc may include beside the skin also the connective tissue of internal organs like gastrointestinal tract, lungs, kidneys, blood vessels and exocrine glands. According to the recent American College of Rheumatology (ACR) / European league against rheumatism (EULAR) classification, there are three subtypes of SSc: SSc without skin involvement, diffuse cutaneous SSc (dcSSc) and limited cutaneous SSc (lcSSc) [1]. In dcSSc, skin sclerosis involves the trunk and the area proximal to the elbow or knee, while in lcSSc skin sclerosis is restricted to face, forearms, hands, lower legs and feet. LcSSc was formerly also known as CREST syndrome and the acronym ‘CREST‘ refers to the five main features [2]:

- Calcinosis
- Raynaud‘s phenomenon
- Esophageal dysfunction
- Sclerodactyly
- Telangiectasia

While patients with CREST syndrome had not to fulfill all five syndrome features and those features were probably never meant to serve as criteria for a specific subtype of SSc, the term ‘CREST syndrome’ was confusing so that it was implemented into the term ‘lcSSc’ by LeRoy et al. [3] in a prior classification. To date, the ACR/EULAR classification by van den Hoogen et al. [1] is widely accepted.

10.1.2 Epidemiology

SSc is a rare disease with a current estimated globally incidence ranging from 8 to 56 cases per million population per year and a prevalence of 38 to 341 cases per million [4]. The rarity of this disease and consecutively scarce epidemiological studies as well as changing classification criteria over the time resulted in a large variability of incidence and prevalence.

Ssc can be found in all ethnic groups and various geographic areas with differences regarding incidence and prevalence. The lowest incidence of SSc appears to be in Asia followed by South America (approximately 11.3 and 16.5 cases per million population per year, respectively). Europe and North America have higher incidence rates (27 and 56 cases per million population per year, respectively). Similarly, prevalence rates seem to be higher in Europe and North America (94-341 and 135-184 cases per million, respectively) than in South America and Asia (105.6-238 and 38-77.7 cases per million, respectively) [4]. Incidence and prevalence rates vary also between different regions of the same continent. There are higher incidence and prevalence rates in Argentina or northern Italy than in Brazil or Poland [5-8]. Further epidemiological data of different countries are listed in table 1. The highest prevalence for SSc was reported in Choctaw Native Americans with 469 cases per 100,000 [9]. For Austria, there are no epidemiological data available yet.

Author	Country/region	Incidence Per million/year	Prevalence Per million
Radić et al. [10]	Croatia, southern Croatia	-	156
Gottschalk et al. [11]	Dominican Republic	-	9.3
El Adssi et al., Meyer et al. [12,13]	France	-	132.2-228.4
Anagnostopoulos et al. [14]	Greece	-	600
Bajraktari et al. [15]	Kosovo	28	146.1
Hoffmann-Vold et al. [16]	Norway, south-east Norway	-	99
Sipek Dolničar et al. [17]	Slovenia	26	-
Kang et al. [18]	South Korea	8	77.7
Andréasson et al. [19]	Sweden, southern Sweden	19	305
Kuo et al., Yu et al. [20,21]	Taiwan	10.9-15	38-66.3
Furst et al. [22]	United States of America	56	135-184

Table 1: Incidence and prevalence rates of SSc.

The most common subtype of SSc is lcSSc with about 50-60% followed by dcSSc with 35-40% and SSc without skin involvement with about 5%. Disease onset of SSc is frequently between 25-55 years although it can occur also in infants and in elderly people. Like several

other connective tissue disorders, SSc has a gynecotropism affecting predominantly females with a female-male ratio of 4:1 in dcSSc and 10:1 in lcSSc while the underlying cause for this predominance is unknown [23]. Although SSc is more common among women, male sex seems to be associated with a higher risk developing disease-related complications and with shorter survival. Further independent predictors of mortality are older age at onset, presence of dcSSc, and several disease-related complications like digital ulcers, renal and pulmonary involvement [24]. Due to sclerosis of internal organs with potential life-threatening complications, SSc generally goes along with a high mortality. Meta-analyses could demonstrate a standardized mortality ratio of 3.53 (95% CI 3.03-4.11) and a cumulative survival from diagnosis of 74.9% at 5 years and 62.5% at 10 years. Death was related to SSc complications in about 58% of deceased patients with SSc while pulmonary involvement and its complications representing to date the leading cause of SSc-related death [25-27].

10.1.3 Etiology

As an autoimmune disorder, the underlying cause leading to SSc is yet unknown. However, there is evidence that several environmental factors, genetic susceptibility and epigenetic modifications are associated with SSc and may trigger vasculopathy, autoimmune pathways and fibrosis leading thus to this disease [28].

10.1.3.1 Environmental factors

Exposure to several environmental factors has been identified contributing to the development of SSc, although a definitive causative role of these factors is still lacking. Especially, occupational exposure to crystalline silica and solvents, like aromatic solvents, white spirit, ketones, trichloroethylene, and chlorinated solvents, seems to have an impact in the development of SSc [29]. A meta-analysis by Rubio-Rivas et al. [30] demonstrated that exposure to silica and solvents is most likely related to SSc (OR 2.81, $p<0.001$; OR 2.00, $p=0.001$, respectively). Additionally, there was also a significant association of epoxy resins ($p<0.001$) while exposure to pesticides and welding fumes did not show significant results ($p=0.90$; $p=0.64$, respectively). Furthermore, an association between SSc and occupational exposure to several heavy metals, like antimony, cadmium, palladium, zinc, mercury, lead and molybdenum, was observed [31]. Moreover, there are limited data that some chemotherapeutic drugs, including taxanes or bleomycin, as well as cytomegalovirus may also

trigger SSc [32-34]. Dietary compounds, like L-tryptophan, other pharmacologic substances, like pentazocine or bromocriptine, and other compounds, including contact lenses, silicone breast implants or hair dyes have also been implicated in the development of SSc. However, no associations of these compounds and substances could be found [29,35]. Smoking does also not increase the risk of developing SSc although it increases the morbidity and mortality in patients with SSc [36].

10.1.3.2 Genetic susceptibility and epigenetics

Genetic studies in patients with SSc identified factors contributing to the development of SSc while the overall genetic burden seems to be moderate as only 2.6% of siblings of patients with SSc suffer also from this disease [37]. Additionally, a study with 42 twin pairs among which at least one twin had SSc revealed a genetic heritability for SSc of only 0.008%, a low overall concordance rate of 4.7% and the concordance was similar between monozygotic and dizygotic twins [38]. On the other hand, siblings of patients with SSc have a 10- to 27-fold increased relative risk and first-degree relatives have a 10- to 16-fold increased relative risk developing SSc [38]. Additionally, several susceptibility genes and loci could be identified being associated with SSc, especially alleles in the human leucocyte antigen (HLA) system or major histocompatibility complex like in other autoimmune disorders. Arnett et al. [39] identified a strong association of SSc to HLA DRB1*1104, DQA1*0501, DQB1*0301 haplotypes and DQB1 alleles encoding a non-leucine residue at position 26 (DQB1 26 epi) in Caucasian and Hispanics as well as to HLA DRB1*0804, DQA1*0501, DQB1*0301 alleles in African Americans. No differences of the associated alleles could be found between dcSSc and lcSSc. Additionally, a negative correlation could be found for DRB1*0701, DQA1*0201, DQB1*0202 haplotypes and DRB1*1501 haplotype alleles which exert a possibly protective role in dominant and recessive models. Genome-wide association studies could identify several single nucleotide polymorphisms located on the HLA-DQB1 gene, as well as on PSORS1C1, TNIP1, RHOB, CD247, MHC, IRF5, and STAT4 genes [40,41]. So far, more than 40 non-HLA loci could be yet identified revealing an association with SSc, including ATG5, CSK, DNASE1L3, IRF8, SCHIP1-IL12A, TNFAIP3, TNFSF4, BANK1, GRB10, JAZF1, KCNA5, PXK and SOX5 genes [37,42].

Overall and despite many identified genes, the impact of genetic susceptibility in the etiology of SSc is yet controversial. Beside genetic susceptibility, epigenetic factors are attributed to play a role in the development of SSc. It has been suggested that epigenetic modifications like

histone modification, changes in micro ribonucleic acid (RNA) and methylation of deoxyribonucleic acid (DNA) may represent the missing link between genetics and environmental factors [43-46]. Histone modification may affect gene expression by changing the chromatin structure while DNA methylation inhibits DNA transcription. Micro RNA are non-coding RNA sequences mediating post-transcriptional regulation of different gene expression by preventing translation or initiating transcript degradation. Micro RNA modulates both, DNA methylation and histone modification, and vice versa [43,45]. In SSc, all three respective epigenetic modifications have an impact on the etiopathogenesis. DNA methylation has an influence on endothelial cells by affecting the gene expression of endothelial nitric oxide synthases (eNOS) and bone morphogenic protein receptor II leading to impaired angiogenesis and increased apoptosis [43]. On lymphocytes, DNA methylation of CD4+ T-cells, CD70 and CD40 ligand is reduced leading to overexpression. CD40 ligand is encoded on the X chromosome which may contribute the female predisposition of SSc [43,45]. On fibroblasts, DNA methylation of FLI-1, a transcription factor which diminishes collagen synthesis of fibroblast, is in contrast increased promoting on that way collagen synthesis and accumulation in SSc [45,47]. Histone modification also contributes to a repression of FLI-1 and to an expression of eNOS [47,48]. In SSc, several micro RNA sequences like miR-21 are upregulated while other like miR-29 and miR-145 are downregulated [43]. These sequences as well as other micro RNA sequences like miR-31, miR-92a and miR-146 are associated with SSc although not all correlated with disease activity [45,49]. The stimuli leading to epigenetic modifications are, however, unclear while environmental factors like exposure to silica, chemical compounds and toxins as well as ageing and oxidative stress may act as trigger factors [45].

10.1.4 Pathogenesis

The pathogenesis of SSc is complex and not yet fully understood. Endothelial cells, platelets, lymphocytes, monocytes and fibroblasts building a pathophysiological network, in which several cytokines, growth factors and adhesion molecules are released resulting subsequently in the three hallmarks of SSc-related pathogenesis: vasculopathy, autoimmune activation and tissue fibrosis (Figure 1). These changes contribute to ischemia, inflammation and dysregulated repair of the connective tissue with fibrosis [28,50].

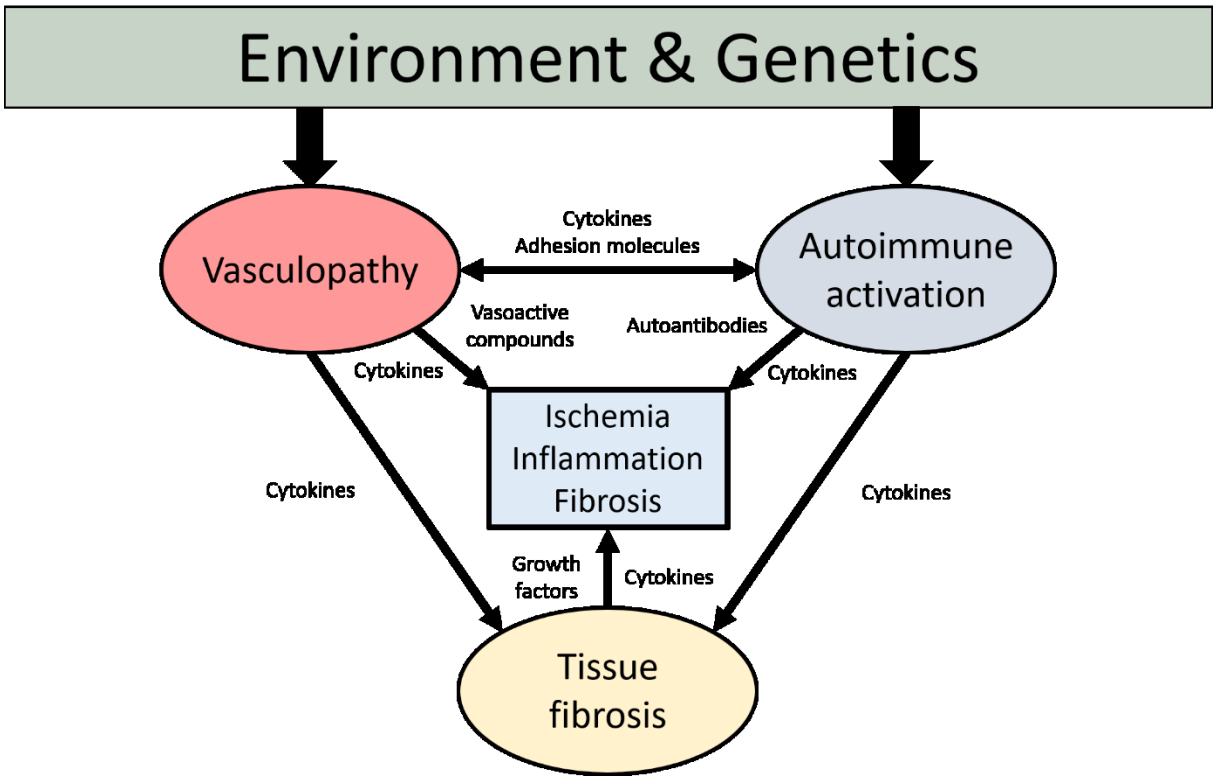

Figure 1: Pathophysiological network of SSc involving vasculopathy, autoimmune activation tissue fibrosis, modulated cytokines, adhesion molecules, vasoactive compounds, autoantibodies and growth factors leading to clinical consequences (ischemia, inflammation, fibrosis). Figure created by Philipp Jud.

10.1.4.1 Vasculopathy

Vascular pathology is a universal finding in SSc including endothelial dysfunction as well as vascular damage with apoptosis of endothelial cells. Vascular injury involves predominantly the microcirculation and smaller arterioles, which are triggered by environmental factors and potentially also by cold exposure, oxidative stress and consecutive hypoxic damage [50,51]. Endothelial cells are already affected in the very early stage of this disease [52]. Large gaps between endothelial cells lead to a loss of integrity of the endothelial lining and a vacuolization of endothelial cell cytoplasm occurs as a sign of the early, edematous stage of vascular injury [53]. Additionally, intimal proliferation with infiltration of macrophages, T- and B-cells occurs. Intimal proliferation as well as accumulation of collagen and glycans resulting in arterial malfunction and arterial stenosis or occlusions [52]. In a later stage of vascular injury, apoptosis of endothelial cells occurs probably due to ischemic injury caused by arterial stenosis and

occlusions and due to humoral and cellular immune factors, including antibody-dependent cellular cytotoxicity, cytotoxic T-cells and anti-endothelial-cell antibodies (AECA) [52]. Apoptotic endothelial cells may perpetuate tissue and vascular damages by activation of procoagulant pathways and the alternate complement system as well as by phagocytosis via immature dendritic cells leading subsequently to an autoantigen presentation of the phagocytized apoptotic endothelial cells in CD8+ T-cells. Thus, autoimmune processes are further promoted [54-56].

Beside these morphological changes of the microvascular structure, vasculopathy leads also to functional disturbances and alterations of physiological activities of the endothelium in SSc. Several compounds mediating vascular tone, migration of inflammatory cells and coagulation processes are involved in the pathogenesis (Figure 2). Nitric oxide (NO) and endothelin have reciprocal effects on the vascular tone. While NO leads to a vasodilation, endothelin is a potent vasoconstrictor. In SSc, vasodilatory effect of NO is impaired, which is related to a decrease of eNOS and a diminished release of NO [57,58]. Furthermore, an impairment of NO release may contribute to intimal proliferation, adhesion and immigration of inflammation cells, platelet aggregation, and modulation of other compounds involved in endothelial cell activation [52,59]. On the other hand, endothelin levels are increased in SSc and more endothelin receptors are expressed on the cell surface of several disease-related organs [60,61]. Additionally, endothelin may contribute also to an enhanced tissue fibrosis by stimulating fibroblast proliferation and synthesis of types I and III collagen [62]. Vascular adhesion molecules, like intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1), E-selectin and P-selectin, mediate the migration of lymphocytes into the vessel wall. Elevated serum levels of ICAM-1, VCAM-1 and selectins were found in patients with SSc which were also positively correlated with the expression of these molecules on the surface of endothelial cells and fibroblasts as well as with disease activity [63]. Several molecules involved in platelet activation and coagulant processes are also altered. Platelet activation is generally amplified in SSc probably secondary to endothelial dysfunction [50,52]. β-Thromboglobulin and platelet factor 4 are strong chemoattractants for fibroblasts which are released by activated platelets and which are elevated in SSc. Additionally, both are procoagulants [64]. Other procoagulant factors, like von Willebrand factor (vWF), vWF multimers, and thrombin-antithrombin complexes, were also significantly increased while fibrinolysis was diminished with objectified decreased levels of plasminogen-activator inhibitor [65]. Elevated vWF was further associated with pulmonary arterial hypertension (PAH) in SSc and, in addition, a decreased level of angiotensin-converting enzyme (ACE) has been suggested playing also a role in SSc, predominantly in PAH [66]. Another protein affecting endothelial cells in SSc is vascular

endothelial growth factor (VEGF). Especially, the subtype VEGF-A stimulates angiogenesis by proliferation and migration of endothelial cells. Increased levels of VEGF and upregulated VEGF receptors have been identified in SSc [67]. However, despite an elevation of VEGF and its receptors, angiogenesis seems to be insufficiently in SSc which may be caused by alternative splicing of the VEGF-A pre-RNA leading to different isoforms of VEGF-A. VEGF-A165a acts pro-angiogenic, pro-fibrotic and promotes neovascularization while the isoform VEGF-A165b acts anti-angiogenic and anti-fibrotic leading to capillary loss. This different isoform expression may have a relevant impact on the vasculopathy of SSc [68].

As a consequence, microvascular structural and functional changes are associated with several symptoms and clinical manifestations of SSc, like abnormalities of capillaries, Raynaud's phenomenon, digital ulcers and PAH [50,52,66,68,69].

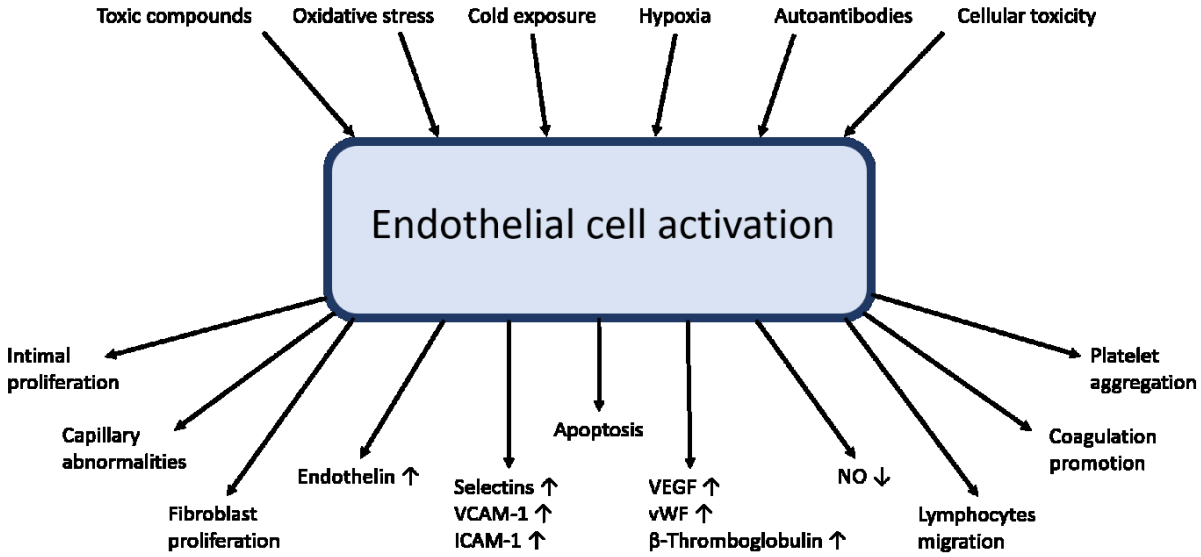

Figure 2: Various mechanisms affecting endothelial cells leading to an activation or to apoptosis of endothelial cells. Figure created by Philipp Jud.

10.1.4.2 Autoimmune activation

Autoimmune activation with subsequent inflammation represents the second hallmark in the pathogenesis of SSc. Many genetic and epigenetic alterations contributing to SSc regulate the immune system and the immune system modulates in turn changes of vasculopathy and tissue fibrosis. A broad spectrum of activated inflammatory cells is present in the perivascular tissue

and various autoantibodies as well as cytokines are released contributing to microvascular damage and tissue fibrosis (Figure 3).

Within cellular immunity, monocytes, macrophages and lymphocytes are primarily involved. Monocytes and macrophages form together with T-lymphocytes perivascular infiltrates. After differentiation from monocytes, macrophages can be classified in classically activated and alternatively activated macrophages regarding their surface markers [70]. Classically activated macrophages produce pro-inflammatory cytokines like interleukin (IL)-1, IL-6 and tumor necrosis factor (TNF)-α. They also express different toll-like receptors and costimulatory proteins, like CD80 or CD86, which promote inflammation in the early stage of SSc. On the other hand, alternatively activated macrophages produce anti-inflammatory cytokines like IL-4, IL-10 and IL-13. They express macrophage scavenger receptors and mannose receptor-1, which promote tissue repair but also tissue fibrosis in skin and internal organs [71-73]. In SSc, higher values of circulating classically and alternatively activated macrophages were observed which may contribute to especially pulmonary changes. Furthermore, patients with lung involvement in SSc feature distinct gene signatures related to macrophage phenotypes [74-76]. Migration and activation of T-lymphocytes, mainly CD4+ T-cells, are enhanced in SSc and CD4+ T-cells are predominantly located in the perivascular tissue [77,78]. Apoptosis of endothelial cell has been suggested to be promoted by cytotoxic T-cells via granzyme/perforin complex. Granzymes are serine proteases which are stored and released by natural killer cells and cytotoxic T-cells. Together with perforin, another cytolytic protein stored in natural killer cells and cytotoxic T-cells, granzymes form a complex mediating apoptosis. Granzymes reactivity and granzyme gene expression were present in SSc [79,80]. Furthermore, subsets of γδ T-cells, which seem to be crucial in the pathogenesis of SSc due to their antigen-presenting capacity and pro- or anti-inflammatory regulatory effects, were pathologically altered with increased percentages of CD27+γδ T cells expressing granzyme B and with upregulated gene expression of granzyme A [81,82]. Decreased levels of regulatory T-lymphocytes could be also observed promoting autoimmune activation while profibrotic T-helper-17 cells are upregulated leading to an increase of IL-17 [83-85]. Additionally, a specific T-cell subtype called angiogenic T-cells, which contribute to angiogenesis and repair of damaged endothelial cells, were increased in patients with SSc, particularly in those patients with digital ulcers [86]. B-lymphocytes contribute further in the pathogenesis of SSc. B-cell activating factor is increased correlating with the severity of skin sclerosis [50]. CD19, a crucial cell-surface protein regulating B-cell activation and differentiation, is overexpressed and CD19 signaling pathway is upregulated in SSc. Both alterations may lead to increased autoantibody

production, chronic hyperreactivity and autoimmunity. Additionally, abnormal B-cell activation may be associated with enhanced fibrosis of skin and internal organs [87-89].

Besides those cellular changes, alterations of the humoral immunity are also present in SSc. Several autoantibodies are produced by B-lymphocytes targeting against extracellular and nuclear antigens. Antinuclear autoantibodies (ANA) and extractable nuclear antigens (ENA) are present among over 95% of patients with SSc [90]. Several ANA subtypes are specific for SSc, such as anti-centromere autoantibodies (ACA), including centromere protein B antibodies (anti-CENP-B) or antitopoisomerase I autoantibodies (ATA), formerly known as anti-Scl-70 antibodies. Less frequent are autoantibodies subtypes against RNA polymerase I, II and III (anti-RNAP I, II, III), antibodies against 70 kDa A and C polypeptides of U1 snRNP (anti-U1-RNP), antibodies against PM-Scl-100 and PM-Scl-75 proteins of the human exosome (anti-PM-Scl) and antibodies against H1/8-2 and Th/7-2 RNA (anti-Th/To), which are components of RNase P and RNase MRP. The production of these specific autoantibodies is usually exclusive. If an autoantibody subtype is present, it rarely changes to another subtype [91]. The presence of these antibodies varies between different subtypes of SSc. ATA are present in approximately 40% of patients with dcSSc, but in less than 10% of patients with lcSSc [92,93]. On the other hand, ACA are more frequent in lcSSc [94]. These autoantibodies represent important diagnostic markers for SSc and are additionally associated with the clinical progress of this disease. Digital ulcers occur more frequently in patients with elevated ACA and ATA. PAH is also predominantly associated with ACA while pulmonary fibrosis occurs more frequent with positive ATA and less frequent with positive anti-RNAP III. Furthermore, anti-RNAP III is highly associated with renal crisis and malignancies [95-97]. Moreover, ANA subtypes have also an influence on the mortality. ACA positivity is associated with a better prognosis and lower mortality in patients with lcSSc than positive anti-Th/To [95]. In addition to ANA, several other autoantibodies were observed in SSc. AECA occur in up to 86% of patients with SSc which are directed against different antigens of endothelial cells [98]. Binding of AECA on antigens leads to endothelial cell activation with a release of vascular adhesion molecules and endothelin as well as cytotoxicity with consecutive apoptosis of endothelial cells [99]. It has been implicated that AECA are associated with severe vascular involvement [100]. Interestingly, the main target of AECA in lcSSc seems to be centromere protein B while AECA bind primarily to endothelial topoisomerase I in dcSSc suggesting that ANA subtypes may act as AECA [101]. Other autoantibodies which are directed against tyrosine kinase receptor, G-protein-coupled receptor, platelet-derived growth factor (PDGF) receptor, angiotensin II type 1 receptor and endothelin type A receptor have also been identified in SSc. The last two

autoantibodies are additionally associated with the occurrence of digital ulcers and PAH [100,102].

Cytokines represent key regulators of immune pathways and play another central role in the humoral immunopathogenesis of SSc. Numerous cytokines including IL, TNF, chemokines and interferons (IFN), which are involved in cell signaling, are pathologically altered. Pro-inflammatory and pro-fibrotic IL, like IL-1α, IL-1β, IL-12, IL-17, IL-18, IL-23 or IL-33, mediate lymphocyte activation and are elevated or upregulated in patients with SSc [103-105]. IL-6 is another pro-inflammatory and pro-fibrotic IL which mediates especially acute phase response. It can be upregulated by tissue damage and also by IL-1. In SSc, IL-6 seems to be a key mediator of the altered cytokine network as IL-6 levels are elevated and associated with skin involvement, pulmonary fibrosis and mortality [103,106,107]. IL-4 and IL-13 act as anti-inflammatory IL on the promotion of the Th-2 response, but contributing to tissue fibrosis as well. Both are elevated in SSc although their participation in inflammation and fibrosis needs to be further elucidated [103,108-110]. Further IL, like IL-10 and IL-22, have similar effects. IL-10 and IL-22 have anti-inflammatory properties on the immune system but promoting to fibrogenesis [111,112]. TNF-α stimulates acute phase response, phagocytosis and the expression of several other chemokines. Elevated levels of TNF-α could be found in patients with SSc and were associated with pulmonary fibrosis and PAH [113,114]. Levels of TNF-related apoptosis-inducing ligand, a member of the TNF superfamily which stimulates apoptosis, were also higher in patients with SSc compared to healthy subjects and were associated with a pulmonary involvement in SSc [115]. Several chemokines of the CC subfamily, including monocyte chemotactic protein (MCP)-1, MCP-3, macrophage inflammatory protein 1α and CCL5, as well as CXCL-4, which is a member of the CXC subfamily, were increased in patients with SSc and correlated with skin and pulmonary fibrosis as well as with PAH. Furthermore, CXCL4 stimulates endothelial cell activation and influx of inflammatory cells [116-118]. Finally, patients with SSc exhibit a distinct transcript pattern including dysregulated IFN-inducible genes [119]. This ‘IFN signature’ leads to an increased production of type I IFN including IFN-α, which is associated with pulmonary fibrosis and digital ulcers [120]. Additionally, IFN-γ, which regulates collagen synthesis and acting anti-fibrotic by inhibition of expressed pro-fibrotic genes, is reduced. Thus, tissue fibrosis will be enhanced [121].

However, although several implications could be identified, which were caused by the altered cytokine network in SSc, the exact interactions and roles of cytokines are not fully understood due to this large number of involved cytokines and many different effects on various cells.

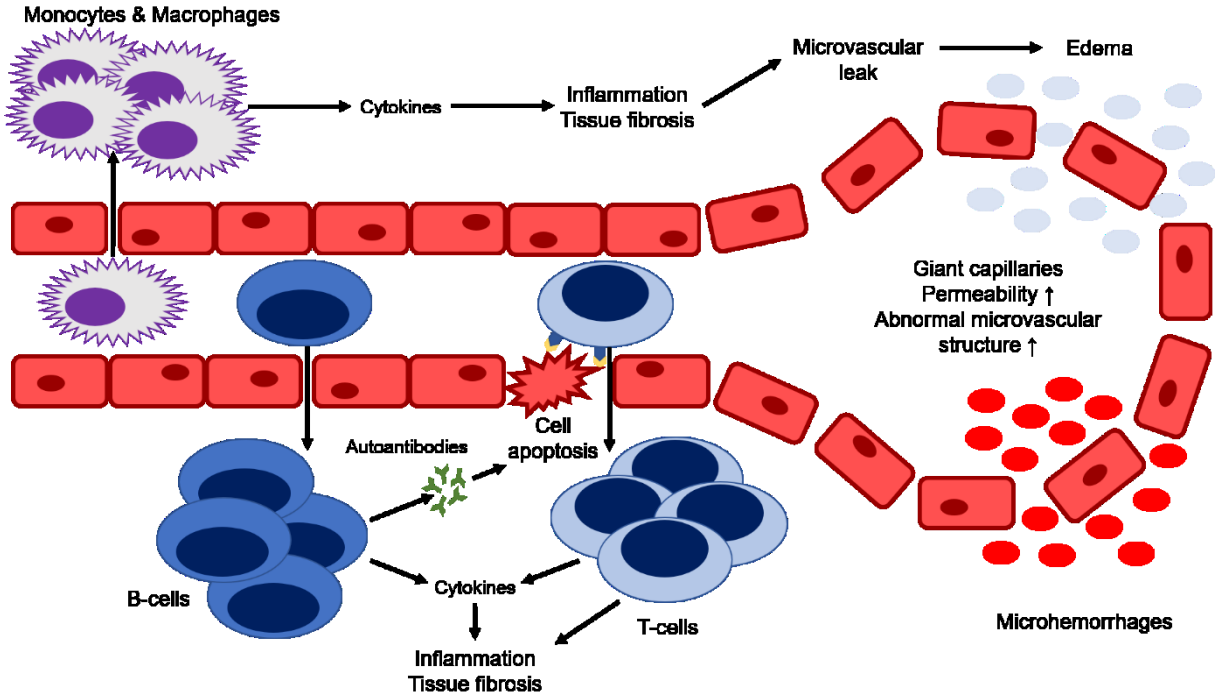

Figure 3: Infiltration of inflammatory cells in the perivascular space and its consequences. Macrophages, T- and B-lymphocytes produce cytokines and autoantibodies promoting to microvascular damage and initiating tissue fibrosis. Figure created by Philipp Jud.

10.1.4.3 Tissue fibrosis

Tissue fibrosis occurs in the late stage of SSc as a consequence of vasculopathy and autoimmune activation. Activation of tissue cells and release of pro-fibrotic mediators leading to deposition of extracellular matrix components, which cause further dysfunction of the affected tissues and organs.

The fibrotic tissue is characterized by a large number of myofibroblasts. The differentiation and proliferation of myofibroblasts is contributed by several other cells including fibrocytes, adipocytes, pericytes and endothelial cells in a pro-fibrotic milieu including cytokines, like IL-6 or IL-13, growth factors and other mediators. As a consequence, fibroblasts, which are differentiated from myofibroblasts, are activated and extracellular matrix proteins, like collagens, glycosaminoglycans, elastin, fibronectin, and tenascin, are increasingly secreted [100]. The progressive accumulation of extracellular matrix proteins in skin and organs resulting in a reduced elasticity and in mechanical stress of the tissue. Additionally, those extracellular matrix proteins can be alternatively spliced leading to isoforms, like fibronectin

containing extra domain A or tenascin-C. Both, reduced elasticity with mechanical stress and isoforms of extracellular matrix proteins, upregulate in turn fibroblast activation and fibrosis progression in SSc [122-124]. Furthermore, the fibrotic process is perpetuated by a failed termination of the physiologic tissue repair to the inflammatory stimulus and by epigenetic changes and altered micro RNA expression of fibroblasts, which may contribute to a persistent activation phenotype of fibroblasts [125-127].

Another pivotal role in fibrosis development seems to be an endothelial- and epithelial-to-mesenchymal transition. The endothelial-to-mesenchymal transition is a differentiation process whereby endothelial cells lose their morphology and expression of physiological phenotype markers and obtain features of mesenchymal cells or myofibroblasts including the expression of mesenchymal cell products. The same process occurs in epithelial cells by the epithelial-to-mesenchymal transition [100,128,129]. This cellular trans-differentiation process seems to be mediated by growth factors, like transforming growth factor (TGF)-β, and vasoconstrictor molecules, like endothelin-1, as a result of preceding endothelial dysfunction and may contribute to abnormal microvascular architecture and to a source of new myofibroblasts in the damaged tissue [129-131]. Furthermore, adipocyte-to-myofibroblast transition, another cellular trans-differentiation process, has been reported and may play also a role in tissue fibrosis of SSc [132].

In addition to cellular changes, several pro-fibrotic cytokines and growth factors are involved in tissue fibrosis. As stated above, there are IL, including IL-4, IL-6, IL-10, IL-13 and IL-22, and TNF-α with pro-fibrotic properties contributing to skin and pulmonary fibrosis [103,106-114]. Among growth factors, TGF-β has a pivotal role. TGF-β include three isoforms which interact with their specific receptors TGFR-1 and TGFR-2 and activating thus small mothers against decapentaplegic homologues (SMAD) 2- and SMAD3-mediated intracellular signaling pathways. Thus, the transcription of pro-fibrotic genes is induced [131,133]. TGF-β is kept in a latent form intracellularly to a propeptide which is further bound to TGF-β-binding proteins creating a so called large latent complex. The connective tissue contains a significant amount of these large latent complexes. Several mediators including integrins, proteases, or thrombospondin-1 can activate latent TGF-β and many of those activators are also elevated in SSc [134-136]. All three isoforms of TGF-β are elevated in SSc and TGF-β enhances the synthesis and crosslinking of extracellular matrix proteins, stimulates many further pro-fibrotic mediators, like connective tissue growth factor and cadherin-11, and promotes the expression of genes encoding for pro-fibrotic compounds in SSc [131,137,138]. Additionally, endothelin-1, which contributes to vasculopathy in SSc, is upregulated by TGF-β and it has been reported

that endothelin features also pro-fibrotic characteristics by myofibroblast activation and increased production of fibronectin [100,139,140].

10.1.5 Clinical characteristics

Vasculopathy, inflammation and tissue fibrosis cause a variety of clinical symptoms while the skin is mostly affected in SSc. Raynaud's phenomenon or acrocyanosis are one of the earliest and most common symptoms with a prevalence of 96% in SSc, which may affect acral sites like digits but also tongue, ear lobe, nose or nipples may be affected [141]. While Raynaud's phenomenon is accompanied with a white, painful or painless discoloration typically triggered by cold or stress and may include cyanotic and/or red discolorations too, acrocyanosis describes only a cyanotic discoloration of acral sites [142]. Both are primarily triggered by vasculopathy and disturbed microcirculation in SSc [143]. Telangiectasias affecting predominantly digits, palms and face are also a consequence of vasculopathy while digital ulcers are a combined consequence of vasculopathy, inflammation and tissue fibrosis. Fingertip ulcers, also called rat-bite necrosis, are not uncommon affecting 33-43% of patients with SSc and represent a serious complication in SSc due to slow and poor healing and possible wound infection with potential loss of tissue [141]. Besides these vascular changes, which are apparent on the skin, other skin manifestations may occur in SSc and can be differentiated into skin changes of the edematous stage and in skin changes of the sclerotic stage. In the early edematous stage, inflammation is predominantly present leading to swollen digits, also called puffy fingers. Cutaneous inflammation may be progressive causing itchy erythema of larger skin areas. In further clinical course, tissue fibrosis getting more prevalent leading to skin sclerosis with a varying degree. Due to skin sclerosis, sclerodactyly is a common finding on the digits and microstomia together with perioral radial folds, called tobacco pouch mouth, limited mimics, called mask face, and a sclerotic shortened frenulum are characteristic signs of the facial involvement. Additionally, progressive skin sclerosis may cause subcutaneous calcifications predominantly at acral sites and at the region of large joints, which may extend into deeper skin layers, even up to muscle and joints, causing lipodystrophy, dry skin, alopecia, muscle atrophy and joint contractures [143].

Besides the skin, SSc affects other organs too. The lung and gastrointestinal tract are further commonly affected organ systems in SSc [141]. Pulmonary involvement is often characterized by a decreased diffusion capacity in pulmonary function testing which cause however not necessarily symptoms. Distinct vasculopathy is associated with PAH while tissue fibrosis is

associated with pulmonary fibrosis [52,120]. Both, PAH and pulmonary fibrosis, may cause dyspnea, chest pain, fatigue and general weakness. PAH may lead additionally to palpitations and syncope while pulmonary fibrosis is associated with chronic dry cough. Gut dysmotility is another typical finding presenting with dysphagia and gastro-esophageal reflux by an affection of the esophagus but also with constipation, bloating, diarrhea and malabsorption due to affected stomach, small and large intestine. Complications of gastrointestinal involvement in SSc include peptic strictures and Barrett's esophagus, gastric antral vascular ectasia, gastrointestinal bleeding, bacterial overgrowth and diverticulosis.

Furthermore, heart, musculoskeletal system, exocrine glands and kidneys may be also affected in SSc leading to perimyocardial fibrosis, cardiac arrhythmia and insufficiency, muscle and joint pain, myopathy, arthritis, dryness of mouth and eyes, and renal crisis including arterial hypertension, proteinuria, nephrosclerosis and renal failure [28,141,143,144].

Regarding on the extent of skin involvement and other distinguishing clinical associations, SSc can be classified as lcSSc and dcSSc. Distal limb and facial skin sclerosis occur in lcSSc while skin sclerosis involving proximal limbs or trunk occurs in dcSSc. LcSSc is further associated with a longer history of Raynaud's phenomenon and a later onset of other related symptoms than Raynaud's phenomenon compared to dcSSc (4.8 years vs. 1.9 years, respectively). Additionally, disease manifestations as well as complications are less common in lcSSc than in dcSSc, except isolated PAH which occurs more frequently in lcSSc suggesting that vasculopathy is more pronounced in lcSSc. Therefore, lcSSc is often indicated as the milder subtype of SSc, which is however only partially correct. Although many disease manifestations and complications occur less frequently in the early stage compared to dcSSc, they may develop more commonly and seriously in the later stage in patients with lcSSc [2,28,141].

10.1.6 Diagnosis

Patients with suspected SSc should be further evaluated by specialists to establish the diagnosis. A definite diagnosis can be established by fulfilment of the recent ACR/EULAR classification criteria [1]. These classification criteria are very accurate with a respective sensitivity of 91% and a specificity of 92% so that they can be used for the majority of patients with suspected SSc. A total score is determined by adding all scores of the respective item counting only the higher score of the respective sub-item if more than two sub-items are present. Patients with a total score of ≥ 9 can be classified having definite SSc.

Basic diagnostic work-up in SSc includes medical history asking the patient about possible symptoms of SSc and physical examination looking for specific changes and skin sclerosis. Modified Rodnan Skin Score (mRSS) is an evaluated diagnostic tool to determine the extent and severity of skin sclerosis by testing the skin movability. A high mRSS is associated with a higher mortality [145,146]. Furthermore, laboratory testing including autoantibodies and nailfold videocapillaroscopy (NVC) are also parts of the diagnostic work-up. NVC can visualize by magnifying the capillary morphology of the nailfold. Typically, the nailfolds of the second to the fifth finger are evaluated whereby patients should remove nail polish before the examination. By visualization of the capillaries, the morphology is examined. Physiological capillaries are fine, U-shaped and have a vessel diameter of less than 20µm. In addition, there should be more than nine capillaries within 1mm of the row of the nailfold bed. There are several morphological abnormalities of capillaries, including irregularly enlarged capillaries, giant capillaries, microhemorrhages, neovascularization, ramified or bushy capillaries, tortuous capillaries, thrombotic capillaries, capillary edema, avascular areas and disorganized microvascular array with irregular capillary loop shape. Some of these morphological abnormalities can be summed up to SSc-specific capillary patterns (Figure 4a-d) indicating the current status and progression of microvascular damage. The early pattern is characterized by few giant capillaries and microhemorrhages without avascular areas. The active pattern is defined as the presence of common giant capillaries and microhemorrhages, moderate avascular areas and mild disorganized microvascular array with few ramified capillaries. The late pattern is characterized by irregular enlargement of capillaries with disorganized microvascular array, few or absent giant capillaries and microhemorrhages, but severe avascular areas and many ramified capillaries or neovascularizations [147-149]. Morphological capillary abnormalities, like giant capillaries and active pattern, are associated with the occurrence of digital ulcers in SSc [150].

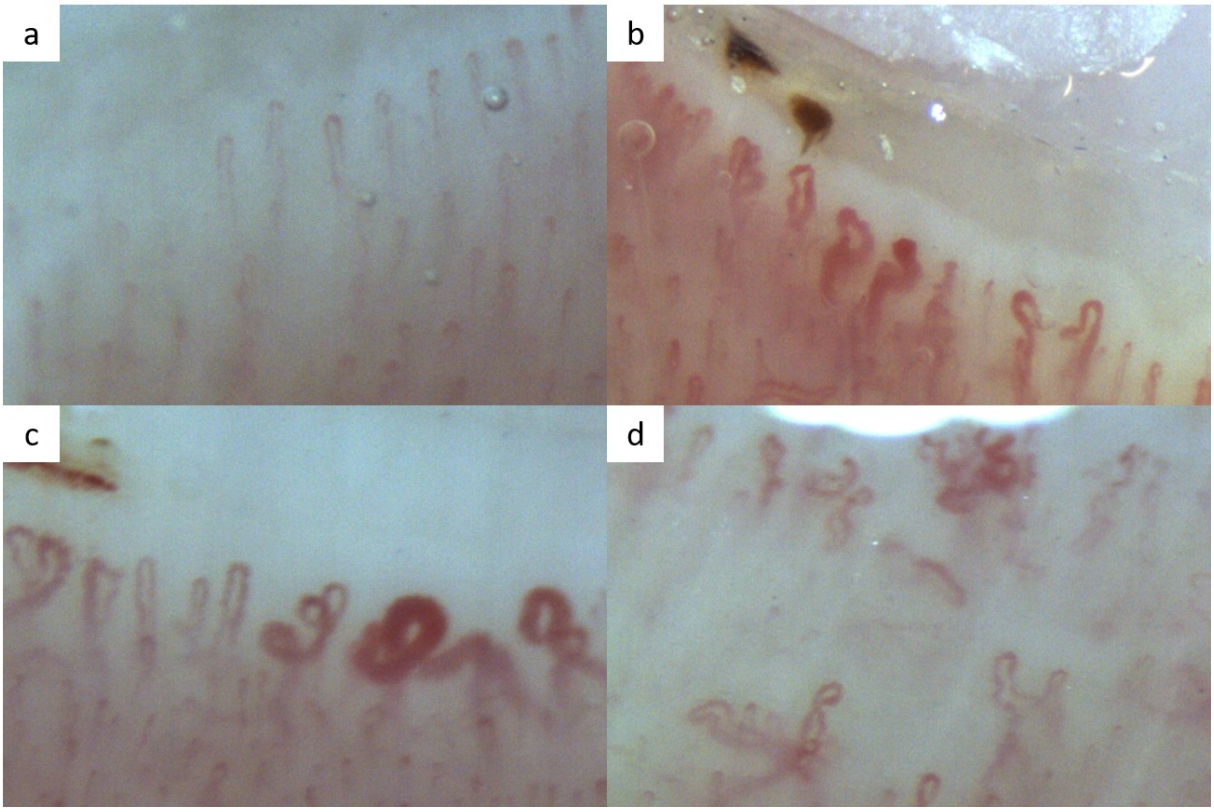

Figure 4a-d: Physiological and SSc-related capillaroscopic patterns. a) Physiological capillary morphology with fine, U-shaped capillaries. b) Early pattern in SSc with a few giant capillaries and microhemorrhages. c) Active pattern in SSc with many giant capillaries, microhemorrhages, thrombotic capillaries and some ramified capillaries. d) Late pattern in SSc with intensive disorganized microvascular array, many ramified capillaries and absent giant capillaries and microhemorrhages. Figure created by Philipp Jud.

Regarding to patient's clinical presentation and symptoms, further imaging methods should be performed to evaluate the presence of possible complications related to SSc. Digital acral plethysmography is a sensitive method to determine the vascular perfusion of digits, whereby the influence of cold and heat on the vascular perfusion can be additionally quantified. In case of inflamed digital ulcers, microbiological smears of the ulcers and X-ray of the bones or even magnet resonance imaging of the respective digit should be performed to identify bacteria and to evaluate potential osteomyelitis. X-rays of digits, hands or other areas may be performed too if subcutaneous calcifications or joint contractures are present. If pulmonary involvement is suspected, echocardiogram, spirometry with diffusing capacity of the lung for carbon monoxide (DLCO), chest X-ray and high-resolution computed tomography should be performed. Pleural ultrasound can be also used to detect pleural changes associated to pulmonary fibrosis [151]. If PAH is highly suspected, pulmonary artery catheterization can

definitely determine existing PAH [152]. Diagnostic work-up for gastrointestinal involvement include disease-related questionnaires like UCLA-SCTC GIT 2.0 questionnaire, barium swallow X-ray, esophageal manometry and endoscopy of the upper and lower part of the gastrointestinal tract to evaluate gut dysmotility and complications [153,154]. Echocardiogram and electrocardiogram (ECG) are used to clarify the extent of cardiac involvement and to exclude other possible heart diseases, while Schirmer's test and Saxon test are able to evaluate xerophthalmia and xerostomia. For renal involvement, renal ultrasound and urinalysis, including urine proteins, creatinine and sediment, should be performed.

10.1.7 Therapy

Since diagnosis of SSc was established, appropriate treatment and follow-up visits should be initiated. Due to the involvement of several organs, the therapy should be adjusted to the underlying symptoms and complications. Currently, two guidelines for the treatment of SSc have been published including pharmacological and non-pharmacological therapeutic options [155,156].

10.1.7.1 Pharmacological therapeutic options

According to the EULAR as well as British Society of Rheumatology (BSR) and British Health Professionals in Rheumatology (BHPR) guidelines, several immunosuppressive agents may be used for skin involvement [155,156]. Methotrexate may be considered in patients with early SSc to improve skin changes (EULAR recommendation A, BSR/BHPR recommendation II,B). Skin involvement may be also treated with other immunosuppressive agents include cyclophosphamide, azathioprine, mycophenolate mofetil, rituximab and oral steroids (BSR/BHPR recommendation III,C), but their efficacy has not been studied extensively. In case of rapidly progressive SSc, hematopoietic stem cell transplantation should be considered while the high risk of treatment-related morbidity and of early treatment-related mortality should be noted (EULAR recommendation A, BSR/BHPR recommendation Ib,B). Evidence for skin calcifications is limited and treatment include antibiotic therapy for infected subcutaneous calcifications as well as surgery for severe calcifications, which restrict quality of life (BSR/BHPR recommendation III,D). For itchy skin, antihistamines may be used (BSR/BHPR recommendation III,C). Interestingly, there are not any recommendations yet available

explicitly for skin involvement in lcSSc, although these recommendations are commonly applied in daily clinical practice also for patients with lcSSc.

Raynaud's phenomenon is a common finding in SSc and calcium channel blockers like nifedipine should be used as first-line therapy (EULAR recommendation A, BSR/BHPR recommendation Ia,A). Other agents including phosphodiesterase-type 5 inhibitors like sildenafil (EULAR recommendation A), angiotensin II receptor blockers (ARB) like losartan (BSR/BHPR recommendation Ib,C), selective serotonin reuptake inhibitors like fluoxetine (EULAR recommendation C), and alpha-blockers like doxazosin (BSR/BHPR recommendation III,C) should be considered, especially in severe cases and if treatment with calcium channel blockers has failed. Intravenous prostanoids reduce also the severity and frequency of Raynaud's phenomenon and may be therefore considered if oral agents have failed (EULAR recommendation A, BSR/BHPR recommendation Ia,B). Possible side effects of these agents include hypotension, leg edema, dizziness, myalgia, nasal stuffiness and headaches which may limit the efficacy and may challenge the treatment of Raynaud's phenomenon. Vasodilatory therapy with phosphodiesterase-type 5 inhibitors and the endothelin receptor antagonist bosentan improve healing of digital ulcers and may prevent the development of new digital ulcers in SSc (EULAR recommendation A, BSR/BHPR recommendation IIa,B and Ia,B, respectively). Therefore, these agents should be considered in the treatment of digital ulcers as well as prostanoids, which are also efficacious in healing of digital ulcers (EULAR recommendation A, BSR/BHPR recommendation Ia,B) [155,156]. In addition, antibiotics may be needed to treat infected ulcers [157].

Pulmonary involvement in SSc represents the main cause for SSc-related death making treatment of PAH and pulmonary fibrosis mandatory [26]. For the treatment of PAH, phosphodiesterase-type 5 inhibitors, endothelin receptor antagonists, riociguat, which a stimulator of soluble guanylate cyclase, and prostanoids should be considered (EULAR recommendation A and B) due to the improvement of exercise capacity and also hemodynamic parameters. Combination therapy of these drugs should be administered in severe PAH cases. Regarding pulmonary fibrosis, cyclophosphamide is recommended, especially in patients with progressive pulmonary fibrosis (EULAR recommendation A, BSR/BHPR recommendation I,A/B). Alternatively, mycophenolate mofetil may be considered in patients with pulmonary fibrosis (BSR/BHPR recommendation II,B) Additionally, hematopoietic stem cell transplantation achieved improvement of lung volumes [158].

Renal crisis is associated with several complications including renal failure. Therefore, blood pressure and renal function should be monitored closely and therapy with ACE inhibitors

should be initiated if renal involvement is present (EULAR recommendation C, BSR/BHPR recommendation III,C). Combination therapy of other anti-hypertensive agents together with ACE inhibitors may be considered in refractory arterial hypertension (BSR/BHPR recommendation III,C). Therapy of gastrointestinal involvement in SSc depending on the present symptoms. Gastro-esophageal reflux should be treated with proton-pump inhibitors and H2-receptor antagonists (EULAR recommendation B, BSR/BHPR recommendation III,C). Prokinetic drugs like metoclopramide may be also used for gastro-esophageal reflux and also for dysphagia, bloating and early satiety (EULAR recommendation C, BSR/BHPR recommendation III,C). In symptomatic bacterial overgrowth, intermittent oral antibiotics are recommended (EULAR recommendation D, BSR/BHPR recommendation III,C). For the management of diarrhea, anti-diarrheal agents like loperamide should be administered, while laxatives may be used for constipation (BSR/BHPR recommendation III,C). Therapy of cardiac involvement depending also on the present form of heart failure. In systolic heart failure, implantation of pacemaker or cardio defibrillator may be considered, especially if arrhythmia is present, (BSR/BHPR recommendation IV,D and BSR/BHPR recommendation III,D) and immunosuppressive agents, carvedilol as well as ACE inhibitors may be administered (BSR/BHPR recommendation IV,D). In diastolic heart failure, diuretics like furosemide may be considered (BSR/BHPR recommendation IV,D). Musculoskeletal involvement should be treated with above-named immunosuppressive agents (BSR/BHPR recommendation III,C) including also TNF inhibitors and tocilizumab [155].

10.1.7.2 Non-pharmacological therapeutic options

Non-pharmacological therapy is widely tolerated and accepted in patients with SSc which may influence disease progression positively and may be therefore considered (BSR/BHPR recommendation III,D).

Physical therapy of hand and fingers, including stretching exercises, occupational therapy, soft tissue massages and local warming with thermal and mud baths, has beneficial effects on the preservation of hand function and mobility. Additionally, pain relief and improvement of Raynaud’s phenomenon could be also achieved [159]. Combination therapy with physical hand therapy and paraffin wax baths suggesting additional improvement in hand mobility and skin elasticity [160, 161]. Phototherapy with ultraviolet A irradiation or photochemotherapy with psoralen combined with ultraviolet A irradiation may be used to improve skin sclerosis via effects on lymphocyte apoptosis, inhibition of collagen synthesis and increased collagenase

production [162]. Patients with SSc should avoid acral injuries due to the risk of potential evolving digital ulcers. In case of digital ulcers, local debridement and wound care with topical dressings should be performed [157]. Smoking has several negative effects on disease progression in SSc although its impact on skin sclerosis seems to be less distinct than on other disease complications [36,163,164]. Nevertheless, smoking cessation should be favored [165].

Overall, the EULAR and BSR/BHPR guidelines focus on the management of specific organ manifestations in SSc providing several therapeutic modalities. However, general therapeutic approaches, therapeutic algorithms and evidence-based data for second-line therapy are lacking. Additionally, many immunosuppressive agents have been recommended although their evidence is commonly weak.

10.2 Endothelial dysfunction in SSc

Endothelial dysfunction is a general term describing functional disorder of the endothelium. As the endothelium holds many functions in the preservation of vascular physiology, endothelial dysfunction encompasses morphological and functional changes of endothelial cells, alterations of vasoactive compounds and vascular remodeling. Several parameters have been identified indicating endothelial dysfunction including imaging and laboratory parameters.

10.2.1 Flow-mediated dilation

Flow-mediated dilation (FMD) represents an especially endothelium-dependent process reflecting the relaxation of a conduit artery when this artery is exposed to an increased blood flow and therefore to an increased shear stress [166]. Due to this phenomenon, endothelial cells release several vasodilators, like NO, prostaglandins, and endothelium-derived hyperpolarizing factor. Despite the fact that numerous mediators seem to influence FMD, the major component responsible for this shear-stress-related vascular response is NO [167-169]. Specialized endothelial, calcium-activated potassium channels are opened in response to shear stress leading to hyperpolarization of the endothelial cells. Thus, calcium entry is increased which activates subsequently eNOS. Finally, the resulting generation of NO leads to FMD [170]. The release of NO and consecutive arterial dilation are not solely related to the activation of eNOS. Alternate endothelial mediators like prostaglandins may co-contribute in the signal pathway between the endothelium and the smooth muscle cells [171].

FMD is assessed in large peripheral arteries, commonly in the brachial artery, but also in the radial or femoral artery. In order to evaluate endothelial response, it is necessary to provoke a shear stress stimulus at the artery of interest resulting in an endothelial production of NO, typically by a blood pressure cuff inflating to suprasystolic pressure in order to occlude the artery. For the assessment of brachial FMD, the cuff is usually positioned below the antecubital fossa on the forearm. The ischemia-induced reactive hyperemia results in an increased shear stress and consecutive dilation of the artery. This vasodilation is interpreted as NO-dependent vascular response which can be used as a marker of NO-bioavailability. Reduced FMD indicates a low NO-bioavailability. Celermajer et al. [172] firstly described the present technique for the assessment of FMD in 1992. In order to reduce interobserver variability and facilitate comparability between different studies, current guidelines for the assessment of FMD standardized this method [173].

Besides this endothelium-dependent response after provocation of local wall shear stress, it is common routine and important part of any FMD-study to assess the maximal obtainable vasodilator response. This is done using an exogenous NO-donor. Therefore, a single dose of oral nitroglycerine is used in order to provoke endothelial-independent vasodilation or nitroglycerine-mediated dilation (NMD).

10.2.1.1 Clinical significance of FMD

FMD is a validated non-invasive technique for the measurement of macrovascular endothelial dysfunction and is defined as the change in post-ischemic diameter as a percentage of the baseline diameter [174]. Proposed cut-off values of FMD and NMD in healthy people are 7.1% and 15.6%, respectively, while also a range from 7% to 10% of FMD values have been reported as physiological [166,175]. Various studies investigated the role of brachial FMD, which represents the early vascular injury in the pathogenesis of atherosclerosis regarding the risk for cardiovascular disease [172,176-178]. In patients with cardiovascular diseases, an impaired FMD is described. The Framingham Heart Study showed FMD-values of 3.3% in females and 2.4% in males with ranges from 0 to 5% in patients with known cardiovascular disease [179]. Regarding SSc, several studies reported an increased endothelial dysfunction by a reduced FMD and NMD among patients with SSc [180-184].

10.2.2 Aortic pulse wave velocity

Pulse wave velocity (PWV) is the velocity at which the blood pressure pulse spread through arteries. Increased arterial stiffness leads to an increased PWV so that arterial stiffness, structural vascular characteristics and also endothelial dysfunction can be assessed by PWV [185-187]. PWV can be differentiated between central and peripheral PWV. Central PWV, like aortic PWV (aPWV), indicates changes of central elastic arteries while peripheral PWV specifies changes of peripheral muscular arteries, which is in addition higher than in central elastic arteries. Due to physiological changes of the vessel wall with degradation of elastic fibers and replacement by collagen fibers, arterial stiffness and PWV will be increased with ageing. Apart from physiological ageing, numerous diseases are associated with increased PWV, especially aPWV seems to be a strong cardiovascular predictor for cardiovascular events and cardiovascular mortality [185].

APWV can be measured locally, regionally or systemically of important arterial sites, where the arterial buffering function is expressed like brachial, femoral or carotid artery. Besides invasive catheter measurement methods, there are several non-invasive measurement methods of the aPWV including ultrasound, magnet resonance imaging or pulse wave analysis devices. Automatic pulse wave analysis devices include tonometric, oscillometric, volume-plethysmographic, and photo-plethysmographic devices. Via software tools, pulse pressure, ejected and reflected pulse wave can be analyzed and aPWV can be calculated. The time from the onset of the ejected pulse wave to the onset of the reflected wave represents aPWV [185,186]. Measuring the aortic pulse pressure waveform, several other parameters of the arterial stiffness can be derived from it including augmentation pressure and augmentation index (AIx). Augmentation pressure represents the additional aortic systolic pressure generated by returning reflected pulse wave and AIx is defined as the augmentation pressure as a percentage of the aortic pulse pressure [188].

10.2.2.1 Clinical significance of aPWV

As a validated parameter for arterial stiffness and for macrovascular endothelial dysfunction, aPWV was included in the recent guidelines for the management of arterial hypertension of the European Society of Cardiology and the European Society of Hypertension [189]. An increased aPWV of >10m/s indicates organ damage and higher arterial stiffness which could be established in elderly subjects and patients with arterial hypertension or diabetes mellitus

[185,190,191]. Furthermore, increased aPWV is associated with higher incidence of cardiovascular events and cardiovascular mortality in general population and in patients with cardiovascular diseases [192,193]. Increased aPWV could be also identified in autoimmune disease like systemic lupus erythematosus, Sjogren syndrome and SSc, while data on aPWV only in patients with lcSSc are limited [194-196].

10.2.3 Endothelial-derived microparticles

Microparticles are small cell membrane vesicles which are released due to activation or during apoptosis of various cell types, including platelets, endothelial cells, vascular smooth muscle cells, and monocytes. These microparticles contain parts of the respective cell membrane including specific cell receptors and cytoplasmic components. Additionally, the protein and lipid composition vary between different microparticles and also the formation process of microparticles influences microparticle composition [197]. The release of endothelial-derived microparticles (EMP) occurs after activation of endothelial cells by several inflammatory cytokines, bacterial lipopolysaccharides, hypoxia, hyperglycemia or during apoptosis. Activated endothelial cells shed microparticles with featured specific markers like CD54, CD62E or CD106 while microparticles of apoptotic endothelial cells are positive for CD31, CD51, CD105 or CD144 [198]. It must be noted that the relative proportion of CD62E+/CD31+ EMP distinguishes the origin of EMP better than the absolute levels. A CD62E+/CD31+ ratio higher than 10 reflects an origin from endothelial cell activation whereas a value of 1 or less indicates that the EMP originate from cell apoptosis [199]. The mechanisms of microparticle release are not fully elucidated, however, it is assumed that intracellular calcium is increased by cell stimulation which dysregulates membrane balance followed by membrane collapse on the one hand and activation of proteases and Rho kinase leading to cytoskeleton cleavage and rearrangement on the other hand [197]. The shedding of microparticles is associated with a disturbance of the structural cell architecture [200].

10.2.3.1 Clinical significance of EMP

EMP have been associated with endothelial injury and endothelial dysfunction in cardiovascular diseases, like coronary artery disease, cerebrovascular atherosclerosis, PAH and also after organ transplantation [198,201]. EMP play a role in vascular homeostasis, including vascular permeability, angiogenesis, leucocytes activation, and platelet function, by

acting angiogenic, pro-inflammatory, and procoagulant, while different EMP phenotypes affect vascular biology in a different extent [202-204]. CD31+, CD51+ and CD144+ EMP reflect structural endothelial injury while CD105+ EMP contribute to angiogenesis and inflammation. CD54+ and CD62E+ EMP are indicators of leucocytes migration and endothelial inflammation [198,205,206]. EMP have been also associated in SSc while their clinical value remains unclear. On the one hand, significant lower levels of total EMP could be found in patients with SSc compared to healthy controls [207,208]. On the other hand, Michalska-Jakubus et al. [209] reported that total EMP levels are elevated in SSc. However, as mentioned above, a CD62E+/CD31+ EMP ratio seems to be more appropriate than absolute values but data on this ratio are missing yet in SSc. Data regarding EMP phenotypes are scarce while elevated CD143+ EMP and CD144+ EMP have been associated to lcSSc and to PAH respectively, and elevated levels of annexin V non-binding EMP may contribute to pulmonary fibrotic changes and capillary nailfold microangiopathy [209-211].

10.2.4 Arginine, homoarginine and dimethylarginines

Arginine is a semi-essential α-amino acid which is formed in the urea cycle from carbamoyl phosphate, ornithine and aspartate. Arginine is used in the biosynthesis of proteins whereby only L-arginine occurs naturally while its enantiomer, D-arginine, does not occur in proteins. Besides its function in protein biosynthesis, arginine acts as a precursor of NO improving thus NO synthesis and potentially also endothelial function [212]. Arginine can be catabolized into NO and citrulline by eNOS while citrulline can be reutilized to form arginine by sequential reactions via arginosuccinate synthase and arginosuccinate lyase, the so-called arginine-citrulline cycle [213]. Nevertheless, most of the plasma arginine derives from diet and proteolysis of cellular proteins while only a minor fraction is recycled via the arginine-citrulline cycle [214]. Another amino acid deriving from arginine metabolism, but occurring also naturally, is homoarginine. Homoarginine looks chemically similarly to L-arginine discriminating only by an additional methylene group which can be produced endogenously by enzymatic reactions from lysine and arginine by arginine-lysin aminotransferase [215]. Comparably to arginine, homoarginine also serves as a substrate for NOS catabolizing NO and homocitrulline and improving thus endothelial function [216,217]. However, homoarginine seems to be a poorer substrate for eNOS [218]. The methylated arginine compounds asymmetric dimethylarginine (ADMA) and symmetric dimethylarginine (SDMA) are endogenous products deriving also from proteolysis of cellular proteins, whereby both compounds undergo prior enzymatic reactions via protein arginine methyltransferases. ADMA acts as a competitive and potent inhibitor of

eNOS while SDMA impedes the cellular arginine uptake. Subsequently, NO synthesis is reduced and endothelial dysfunction getting promoted [214,219].

10.2.4.1 Clinical significance of arginine, homoarginine and dimethylarginines

As important mediators of the NO metabolism, arginine, homoarginine, ADMA and SDMA are relevant biochemical parameters of endothelial dysfunction. While the relation of arginine to endothelial dysfunction is, however, ambiguous with reported benefits as well as detriments, lower levels of homoarginine seem to be consistently and independently associated with high cardiovascular as well as all-cause mortality, whereas high levels of homoarginine have a protective effect [220-225]. Furthermore, higher levels of ADMA and SDMA are also independent risk factors for cardiovascular outcome parameters in patients with atherosclerotic diseases and in general population, which are additionally associated to other parameters of endothelial dysfunction, like FMD or intima-media-thickness [212,220,226,227]. In SSc, the clinical significance of these compounds is indistinct. Supplementation of arginine seems to have inconsistent effects on digital vascular perfusion, similarly to the results in atherosclerotic diseases [228,229]. Interestingly, there are no data available about the association of arginine to other SSc-related complications or other parameters of endothelial dysfunction in SSc. ADMA level seems to be elevated in patients with SSc which are further associated with PAH and digital ulcers [230-232]. Data about homoarginine and SDMA in SSc are lacking.

10.2.5 Vascular events

As vasculopathy and endothelial dysfunction play key roles in the pathogenesis of SSc, several micro- and macrovascular alterations as well as cardiovascular outcome parameters and non-cardiovascular parameters, which may be triggered by vasculopathy, can be evaluated in lcSSc [50,52,69,233-236]. In this doctoral thesis, following parameters are defined as vascular events (Table 2).

Feeling of xerostomia/xerophthalmia
Signs of PAH
Dyspnea
Right axis deviation
P pulmonale
Right branch block
QTc prolongation > 450ms
Basal right ventricle diameter >42mm
Inferior caval vein diameter >21mm
Estimated right atrial pressure >15mmHg
Right-ventricle-to-left-ventricle (RV/LV) ratio >1.0
Tricuspid annular plane systolic excursion (TAPSE) <20mm
Tricuspid regurgitation velocity >2.9m/s
Tricuspid valve regurgitation
N-terminal prohormone of brain natriuretic peptide (nT-proBNP) > 150pg/ml
Predicted single breath DLCO < 60%
DETECT score step 1
DETECT score step 2
Skin involvement
mRSS
Telangiectasia
Calcinosis cutis
Puffy finger
Sclerodactyly
Acral necrosis
Renal involvement
Estimated glomerular filtration rate (eGFR)
Creatinine
Urine protein/creatinine ratio
Urine albumin/creatinine ratio
Urine immunoglobulin G
Urine α1-microglobulin/creatinine ratio
Urine β2- microglobulin/creatinine ratio

Gastrointestinal involvement
UCLA SCTC GIT 2.0 reflux
UCLA SCTC GIT 2.0 distension
UCLA SCTC GIT 2.0 fecal soilage
UCLA SCTC GIT 2.0 diarrhea
UCLA SCTC GIT 2.0 social functioning
UCLA SCTC GIT 2.0 emotional well-being
UCLA SCTC GIT 2.0 constipation
UCLA SCTC GIT 2.0 total
Microvascular involvement
Impaired acral perfusion
Number of affected fingers with impaired acral perfusion
Early pattern
Active pattern
Late pattern
Giant capillaries
Microhemorrhages
Capillary ramifications
Capillary loss
Capillary edema
Disorganization of microvascular array
Bushy capillaries
Capillaroscopic skin ulcer risk index (CSURI)

Table 2: Vascular events and disease-specific parameters defining vascular events. Table reproduced from [237] and adapted with permission of Clinical and Experimental Rheumatology.

10.2.6 Periodontal inflammation

Inflammatory conditions affecting gingiva and other surrounding tissues of the teeth are generally caused by bacteria and are the result of poor oral hygiene, which may lead to painful chewing, tooth loss and halitosis. Although periodontal inflammation seems to be at first glance a local problem of the periodontium and mouth, periodontitis may be linked to other diseases. Several studies reported periodontal disease as a potential risk factor for cardiovascular diseases, arterial hypertension or rheumatoid arthritis and as a potential promotor of inflammation [238-240]. Furthermore, patients with periodontal disease have a reported distinctive endothelial dysfunction [241,242]. It has been reported, that patients with SSc are also at high risk developing periodontal disease [243-245]. However, data about underlying

pathophysiological pathways or parameters promoting periodontal inflammation in SSc are scarce. TGF-β1, TNF-α and VEGF have been associated in SSc-related periodontal inflammation while the impact of other parameters of endothelial dysfunction are lacking [246,247].

10.3 Study aims

There are only limited data available yet describing specific parameters of endothelial dysfunction in patients with SSc. While most studies have investigated endothelial dysfunction in general SSc with a pooled patient population including patients with dcSSc and lcSSc, only a few studies have evaluated parameters of endothelial dysfunction solely in patients with lcSSc. Furthermore, the correlation of different parameters of endothelial dysfunction between each other and with inflammatory parameters as well as the association between endothelial dysfunction and clinical parameters of SSc were only rarely evaluated in SSc and are lacking in lcSSc.

The aim of this study was to investigate endothelial dysfunction, assessed by FMD, NMD, aPWV, EMP, arginine, homoarginine, ADMA, and SDMA, and the correlations of these parameters between each other, with inflammatory parameters, and with clinical parameters, including vascular events and periodontal inflammation, in patients with lcSSc. Furthermore, potential differences of these parameters within the group of lcSSc patients were also investigated between those with a disease duration of ≤ 7 years and those with a duration > 7 years.

11. Materials and Methods

11.1 Study design and study population

Patients with known lcSSc diagnosed between 1997 and 2019 were screened at the outpatient clinic for vasculitis and related disorders at the division of Angiology of the Medical University Graz for study inclusion in this doctoral thesis. For the specific diagnosis of lcSSc, the ACR/EULAR criteria were used [1]. A group of race- and sex-matched subjects with diagnosed primary Raynaud's phenomenon between 2006 and 2019 at the division of Angiology of the Medical University Graz and with comparable age distribution (± 3 years) was used as controls. The primary endpoint parameter was the difference of FMD between patients with lcSSc and primary Raynaud's phenomenon. Secondary endpoint parameters were present FMD response < 7%, differences of aPWV, EMP, arginine, homoarginine, ADMA and SDMA as well as the correlations of these parameters to vascular events and periodontal inflammation between patients with lcSSc and primary Raynaud's phenomenon.

Inclusion criterium for the group of patients with lcSSc was the presence of diagnosed lcSSc according to the recent ACR/EULAR criteria [1]. Inclusion criterium for the control group was the presence of diagnosed primary Raynaud's phenomenon. Exclusion criteria for both cohorts were age < 18 years, presence of dcSSc, other connective tissues diseases or vasculitis, preexisting 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), 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. Furthermore, subjects with primary Raynaud's phenomenon were not eligible to serve as controls if lcSSc was suspected in prior medical examinations. Age, race, and sex distribution were controlled after 10 and 20 included patients.

11.2 Measurement of endothelial dysfunction

After information and signing of the informed consent form, fasting blood sampling and urine sampling were obtained from the patients followed by completion of two disease-specific questionnaires and medical history. After 5-minutes rest in supine position, ECG, pulse wave analysis and physical examination including mRSS were performed. Subsequently, after

further rest of 5 minutes in supine position, measurement of FMD and NMD followed by transthoracic right heart echocardiogram, digital acral plethysmography and NVC were performed. Further assessment of periodontal inflammation was done by dental and oral examination at the outpatient clinic for Preventive and Operative Dentistry, Endodontics, Pedodontics and Minimally Invasive Dentistry at the Medical University of Graz on the same day. Additionally, spirometry with DLCO was performed at the outpatient clinic for Pulmonology of the Medical University of Graz within two months after study visit. Measurements of FMD, pulse-wave analysis, laboratory parameters and vascular events, except for spirometry and DLCO, were performed between 7:00 am and 1:00 pm after an overnight fast in a temperature-controlled (22-24°C) and quiet room.

11.2.1 Measurement of FMD and NMD

All FMD and NMD measurements were performed by the same trained technician, experienced in performing FMD-measurements, according to the guidelines by Corretti et al. [173] to avoid interobserver variability. The trained technician had an experience of more than 1000 supervised scans prior to initiation of the study and a frequency of more than 500 scans per year in order to assure competency. Vasodilation of the brachial artery was measured with a linear array transducer with 8-13MHz (Siemens ACUSON S2000™, Siemens Healthcare Corp., Henkelstr., 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 centimeters above the ante-cubital fossa by a high-resolution linear array ultrasound transducer. The diameter of the brachial artery was assessed via ultrasound in one segment with definite intimal layers between the lumen and the vascular wall. The distance between the two intimal layers was measured ECG-gated in the end-diastolic phase, which is defined as the beginning of the R-wave in ECG, during the image acquisition in a one-centimeter-long vascular segment at least three times. The position of the transducer was marked on the skin with a water-proof text-marker in order to assure that following measurements were taken from the same vascular segment. Afterwards, the cuff was inflated 50mmHg above the resting systolic pressure of the subject to assure complete occlusion of the artery and to induce shear stress. After an occlusion time of 5 minutes, the cuff was rapidly deflated and the post-ischemic diameter of the initial brachial vascular segment was measured again 45 seconds after cuff release.

After a rest of 20 minutes between FMD-measurement and the baseline-measurement for the NMD-assessment, the brachial arterial diameter was recorded again similar to the technique described for FMD. Subsequently, a single dose of 0.4 mg nitroglycerine was administered sublingually to the subject. Three minutes after nitroglycerin-intake, the brachial arterial diameter was remeasured in the same vascular segment at least three times. All NMD-measurements were performed with the above described ultrasound system (ACUSON S2000™). We defined an increase in post-ischemic diameter of <7% as pathologic FMD and an increase of the vessel diameter 3 minutes after nitroglycerine application of <15.6% as pathologic NMD [175].

Several factors have to be considered in order to perform FMD-measurement adequately, including subject preparation, equipment, image acquisition, staff and experience. Therefore, all FMD- and NMD-measurements were performed in the morning between 8:00 am and 11:00 am after an overnight fast in a temperature-controlled (22-24°C) and quiet room. All subjects had been asked to stop any nitrate and non-nitrate vasoactive medications one day prior to testing, if such medications were present, and to refrain from intense physical activity on the day of testing. Additionally, all subjects had been asked to stop consumption of caffeine, nicotine and fatty substances 8 hours prior to testing. Furthermore, due to the small size of the arterial intimal layer, an adequately powered ultrasound equipment was essential including an ultrasound system with high-resolution linear array probe with 8-13MHz, which was able to record arterial and Doppler measurements for long periods continuously and which was compatible with an ECG-trigger allowing adequate image acquisition and diameter measurement.

11.2.2 Measurement of aPWV

APWV including augmentation pressure and AIx were measured once by the oscillometric device Mobil-O-Graph® (I.E.M. Mobil-O-Graph, I.E.M., Cockerillstr., Stolberg, Germany). After taking a resting ECG, size-adjusted cuff was placed on the right upper arm about 2 to 4 cm above the ante-cubital fossa in supine position and subsequent pulse-wave analysis was performed. The patients were requested not to speak and not to move over the whole pulse-wave analysis.

11.2.3 Biochemical analyses

Fasting blood samples were obtained immediately after information and signing of the informed consent form from the patients with a needle of at least 21 gauges. Blood samples for the assessment of arginine, homoarginine, ADMA, SDMA, and EMP were centrifuged and stored at -80°C until final analysis in August and November 2020. Hemolysis was avoided to prevent falsification of laboratory parameters.

11.2.3.1 Measurement of EMP

After discarding the first 2ml of blood, the blood was collected in 5ml citrate tubes without venous stasis and kept upright. The plasma was centrifuged at 2.500g for 15 minutes at room temperature to obtain platelet-poor plasma. 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. 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) and fluorochrome-labelled anti-human CD31 and CD42b antibodies (Biolegend) 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 as CD31 is expressed on both platelets and endothelial cells, whereas CD42b is restricted to platelets, thus allowing discrimination between platelet-derived microparticles and EMP. A microparticle gate was established using fluorescent 1µm silica beads (Kisker Biotech) for size calibration. Furthermore, immunohistochemical staining for CD51, CD54, CD62E, CD105, and CD144 were used to quantify CD51+, CD54+, CD62E+, CD105+, and CD144+ EMP [237].

11.2.3.2 Measurement of arginine, homoarginine, ADMA, and SDMA

Blood sample for arginine, homoarginine, ADMA, and SDMA were obtained and subsequently centrifuged at 4000g for 10 minutes at 15°C temperature within 1 hour after blood sampling obtainment. The supernatant was collected and divided into aliquots of 1ml, which were stored at -80°C until final analysis by high-performance liquid chromatography with solid phase

extraction and precolumn derivatization firstly described by Teerlink [248] with slight modifications [249]. According to previous reports, the investigated biomarkers can be assumed as stable [250].

11.2.3.3 Measurement of inflammatory and routine laboratory parameters

Besides laboratory parameters of endothelial dysfunction, other laboratory parameters were measured as routine monitoring for patients with lcSSc and primary Raynaud's phenomenon and recording vascular events at a single central lab of the Medical University of Graz in sera and plasma. Following laboratory parameters were measured as inflammatory parameters:

- Leukocytes
- Erythrocyte sedimentation rate (ESR) and C-reactive protein (CRP)
- Immunological parameters, including ANA and ENA screening, cardiolipin- and β-2-glycoprotein-antibodies and lupus anticoagulant

Following laboratory parameters were measured as routine laboratory parameters:

- Erythrocytes, hemoglobin, platelets and subspecies of white blood cells
- Electrolytes, including, sodium, potassium, chloride and calcium
- Renal parameters, including creatinine, urea and uric acid
- Liver parameters, including bilirubin, cholinesterase, alkaline phosphatase, gamma-glutamyltransferase, aspartate transaminase and alanine transaminase
- Heart parameters, including creatine kinase, creatine kinase MB, lactate dehydrogenase, troponine T and N-terminal prohormone of brain natriuretic peptide (nT-proBNP)
- Metabolic parameters including glucose, hemoglobin A1c, high-density lipoprotein (HDL), low-density lipoprotein (LDL), triglycerides, total cholesterol
- Serum proteins and electrophoresis
- Urine analysis, including urine proteins, creatinine and sediment
- Blood coagulation parameters, including prothrombin time and international normalized ratio of prothrombin time, activated partial thromboplastin time (aPTT), activated protein C, protein C and S, and Lupus-aPTT
- Thyroid parameters, including thyroid-stimulating hormone (TSH), triiodothyronine (T3) and thyroxine (T4)

- Bone metabolism parameters, including vitamin D, procollagen type III N-terminal peptide, parathormone and osteocalcin

11.2.4 Measurement of vascular events

The incidence of vascular events was recorded by medical history, physical examination, laboratory parameters, disease-specific questionnaires or scoring systems, spirometry including DLCO and diagnostic imaging at baseline visit.

After obtaining fasting blood samples and urine sampling from the patients, medical history and disease-specific questionnaires were completed by means of a medical doctor and study nurse. Subsequently, ECG was taken (GE MAC 1200 ST, General Electric Germany Ltd., Bleichstraße, Frankfurt, Germany) after 5-minutes rest in supine position from the patients and physical examination including mRSS were performed after assessing aPWV. Transthoracic right heart echocardiogram was performed with microconvex array transducer with 1-4MHz (Siemens ACUSON S2000™, Siemens Healthcare Corp., Henkelstr., Erlangen, Germany) in supine position after assessment of FMD and NMD followed by one measurement of all fingers via digital acral plethysmography (ELCAT Vasoquant VQ4000, ELCAT Ltd, Bgm.-Finsterwalder-Ring, Wolfratshausen, Germany). Subsequently, NVC (Skinview, Optometron Ltd., Oskar-Messterstr., Ismaning, Germany) of the second to fifth finger on both hands was performed in sitting position and one 1-mm-sized image per digit was captured and stored. The incidence of xerostomia, xerophthalmia and gastrointestinal involvement was recorded by medical history and disease-specific questionnaires [153,154]. Skin involvement including the presence of digital ulcers, sclerodactyly, puffy fingers, calcinosis cutis and telangiectasias was recorded by medical history and physical examination. Additionally, all patients underwent an assessment of the mRSS by clinical palpation at 17 defined body areas by the same vascular specialist [146]. As preexisting PAH was an exclusion criterion, signs indicating potential PAH were recorded by medical history, ECG, spirometry with DLCO, right heart echocardiogram and laboratory parameters regarding recent guidelines for the diagnosis for PAH [251]. Spirometry and DLCO were performed according to recent guidelines [252,253]. Dyspnea, presence of P pulmonale, right axis deviation, right bundle branch block and QTc prolongation >450ms in ECG, basal right ventricle diameter >42mm, inferior caval vein diameter >21mm, estimated right atrial pressure >15mmHg, right-ventricle-to-left-ventricle (RV/LV) ratio >1.0, tricuspid annular plane systolic excursion (TAPSE) <20mm, tricuspid regurgitation velocity >2.9m/s or present tricuspid valve regurgitation in echocardiogram, nT-proBNP of > 150pg/ml, and single breath DLCO <60% were defined as signs of PAH (Table 2). Additionally, the

DETECT algorithm was applied to all subjects to select high risk patients for PAH [152]. Renal involvement was recorded by laboratory parameters and urine analysis, including estimated glomerular filtration rate (eGFR), creatinine, urine protein/creatinine ratio, albumin/creatinine ratio, immunoglobulin G, α1 microglobulin/creatinine ratio, β2-microglobulin/creatinine ratio. Digital acral plethysmography and NVC were performed for the assessment of microvascular changes, such as early, active and late pattern, as defined by Cutolo et al. [148]. In order to assess microvascular disease activity, capillaroscopic skin ulcer risk index (CSURI) proposed by Sebastiani et al. [150] was evaluated with pictures of each digital nailfold which were taken during NVC.

11.2.5 Measurement of periodontal inflammation

Periodontal inflammation was evaluated by dental and oral examination with a dentist's mirror and a dental explorer to evaluate pocket depth of periodontal pockets and bleeding on probe (BOP) reported by Mühlemann et al. [254]. Pocket depth was divided into ≤3.4mm indicating no attachment loss and >3.5mm reflecting attachment loss and periodontal inflammation.

11.3 Statistical analysis

In case of continuous variables, patient characteristics were given as means and standard deviation (SD). Median and interquartile range (IQR) or 25-75th percentiles were used to express skewed data. Categoric variables were represented by frequency and percentages.

Student's t-test was used for normally distributed data and exact Mann-Whitney-U test was used for non-normally distributed data. Categoric variables were analyzed by exact Chi-square test. Subpopulation analysis in patients with lcSSc was performed between patients in the early stage of lcSSc (disease onset within ≤ 7 years prior to enrolment) and patients in the late stage of lcSSc (disease onset > 7 years prior to enrolment) to investigate differences of endothelial dysfunction and vascular events between these two subgroups using also Student's t-test, exact Mann-Whitney-U-test or exact chi-square test.

For the quantification of potential correlation between two parameters different correlation coefficients were utilized. Spearman's rank correlation coefficient was used for non-normally distributed parameters and Pearson's correlation coefficient was used for normally distributed

parameters. Correlations of a metric with a dichotomous parameter were assessed with a rank-biserial correlation coefficient for nonparametric variables.

Statistical significance was assumed for p values<0.05 and statistical analyses were performed with IBM SPSS Statistics 26 (Release 26.0.0.0 2019. Armonk, New York, USA: International Business Machines Corporation).

11.3.1 Sample size calculation

Takahashi et al. [182] measured FMD in patients with dcSSc (n = 20), patients with lcSSc (n = 13) and healthy controls (n = 12). They found a FMD value of 6.7 ± 4.0% in dcSSc, 5.3 ± 2.7% in lcSSc and 7.7 ± 2.0% in healthy controls. We assumed that patients with primary Raynaud's phenomenon have similar values in FMD compared to healthy controls. Using a two-sided t-Test, a minimum of 34 patients in each group was necessary to reach a power of 90% to detect a difference of 2.4% (lSSc: 5.3%, Raynaud's phenomenon: 7.7%) assuming a common standard deviation of 3.0%. With this sample size, a sufficient evaluation of endothelial dysfunction in patients with lcSSc could be performed.

11.4 Ethical approval and informed consent

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

12. Results

In total, 89 patients with lcSSc were screened for study participation, while 28 patients were excluded due to one or more exclusion criteria, 22 patients declined study participation and 1 patient died before study visit was performed. Finally, 38 Caucasian patients with lcSSc (36 females, 94.7%; 2 males, 5.3%) with a mean age (± SD) of 57.89 ± 9.22 years and a mean disease duration (± SD) of 7.11 ± 5.78 years participated in that thesis. Additionally, 38 age-, race- and sex-matched controls with a mean age (± SD) of 57.20 ± 8.96 years and a mean disease duration (± SD) of their primary Raynaud's phenomenon of 5.70 ± 3.17 years were also included. 22 patients with lcSSc had a disease duration ≤ 7 years (57.9%) and 16 patients with lcSSc had a disease duration > 7 years (p=0.083). Patient characteristics at study visit are shown in table 3.

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)	57.89 ± 9.22	57.2 ± 8.96	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)	-
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 atherosclerotic coronary artery disease, n (%)	0 (0)	0 (0)	-
Prior atherosclerotic insult, n (%)	0 (0)	0 (0)	-

Prior atherosclerotic peripheral artery disease, n (%)	0 (0)	0 (0)	-
Prior esophageal involvement, n (%)	9 (23.7)	0 (0)	**0.002**
Prior renal involvement, n (%)	2 (5.3)	0 (0)	0.493
Prior dryness of mouth and/or eyes, n (%)	23 (60.5)	0 (0)	**<0.001**
Prior periodontitis, n (%)	18 (47.4)	16 (42.1)	0.650
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
Laboratory values, median (25-75th percentile)			
Hb (g/dL)	13.8 (12.9-14.4)	13.6 (13.2-14.3)	0.719
Platelets (g/L)	246 (215-297)	264 (223-302)	0.561
HbA1c (mmol/mol)	35 (34-37)	37 (34-38)	0.308

Table 3: Patients characteristics at study visit. Table reproduced from [237] and adapted with permission of Clinical and Experimental Rheumatology.

12.1 Parameters of endothelial dysfunction between lcSSc and controls

12.1.1 Differences of FMD and NMD

Between patients with lcSSc and primary Raynaud's phenomenon, no statistical difference was observed for FMD (4.53 ± 3.67% vs. 4.75 ± 2.99%, p=0.775) or for NMD (20.78 ± 9.09% vs. 22.02 ± 7.8%, p=0.303). Additionally, neither FMD (5.29 ± 3.99% vs. 3.48 ± 2.98%, p=0.137) nor NMD values (22.8 ± 11.19% vs. 18.63 ± 5.77%, p=0.345) differ significantly within

the group of patients with lcSSc between those with a disease duration of ≤ 7 years compared to those patients with a disease duration of > 7 years.

12.1.2 Differences of aPWV

Comparably to FMD and NMD, aPWV did not differ between the group of lcSSc and controls (8.26 ± 1.52m/s vs. 8.49 ± 1.67m/s, p=0.662). There was also no difference of aPWV between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years (7.90 ± 1.57m/s vs. 8.76 ± 1.35m/s, p=0.088).

12.1.3 Differences of EMP

Patients with lcSSc had a higher level of CD31+/CD42b- EMP compared to controls, although this result achieved only borderline significance (median 29U/µl [IQR 22-40U/µl] vs. median 24U/µl [IQR 16-31U/µl], p=0.062). Due to the borderline significance, CD31+/CD42b- EMP were also included in further statistical analysis. EMP for CD51+/CD42b-, CD54+/CD42b-, CD62E+/CD42b-, CD105+/CD42b-, and CD144+/CD42b- were undetectable in both groups. There was no difference of CD31+/CD42b- EMP between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years (median 28U/µl [IQR 20-39U/µl] vs. median 30U/µl [IQR 25-41U/µl], p=0.408).

12.1.4 Differences of arginine, homoarginine, ADMA and SDMA

Only one blood sample revealed a slight hemolysis index while the remaining blood samples did not show any hemolysis. Arginine (median 111.9µmol/L [IQR 102.2-122.2µmol/L] vs. median 111.3µmol/L [IQR 100.6-125.4µmol/L], p=0.580) and homoarginine (median 1.64µmol/L [IQR 1.17-2.08µmol/L] vs. median 1.70µmol/L [IQR 1.25-2.11µmol/L], p=0.663) did not differ significantly between patients with lcSSc and controls, while patients with lcSSc had higher values of ADMA (median 0.66µmol/L [IQR 0.58-0.73µmol/L] vs. median 0.63µmol/L [IQR 0.57-0.67µmol/L], p=0.030) and SDMA (median 0.65µmol/L [IQR 0.57-0.76µmol/L] vs. median 0.62µmol/L [IQR 0.54-0.70µmol/L], p=0.025).

No statistically significant differences were achieved for levels of arginine (median 109.8µmol/L [IQR 99.7-119.3µmol/L] vs. median 121.1µmol/L [IQR 106.3-128.9µmol/L], p=0.084),

homoarginine (median 1.86µmol/L [IQR 1.12-2.09µmol/L] vs. median 1.39µmol/L [IQR 1.19-2.83µmol/L], p=0.573), ADMA (median 0.65µmol/L [IQR 0.57-0.74µmol/L] vs. median 0.67µmol/L [IQR 0.61-0.71µmol/L], p=0.946) and SDMA (median 0.63µmol/L [IQR 0.54-0.77µmol/L] vs. median 0.68µmol/L [IQR 0.63-0.74µmol/L], p=0.214) between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years.

12.2 Differences of inflammatory parameters between lcSSc and controls

Leukocytes were not different between patients with lcSSc and controls (median $5.73 \cdot 10^9$/L [IQR $4.95\text{-}6.39 \cdot 10^9$/L] vs. median $5.58 \cdot 10^9$/L [IQR $4.52\text{-}6.48 \cdot 10^9$/L], p=0.455). Neither ESR (median 9mm/h [IQR 4-13mm/h] vs. median 7mm/h [IQR 3-12mm/h], p=0.133) nor CRP (median 1.0mg/L [IQR 0.6-2.8mg/L] vs. median 0.8mg/L [IQR 0.6-1.4mg/L], p=0.109) differ between both groups. Positive ANA were more commonly detectable in patients with lcSSc (94.7% vs. 26.3%, p<0.001) and the group of lcSSc had higher levels of ENA than controls (median 19.5U/mL [IQR 2.8-31.0U/mL] vs. median 0.1U/mL [IQR 0.1-0.1U/mL], p<0.001). Cardiolipin antibodies (median 1.2U/mL [IQR 0.9-2.0U/mL] vs. median 1.1U/mL [IQR 0.7-1.4U/mL], p=0.110), beta-2 glycoprotein antibodies (median 2.5U/mL [IQR 2.1-2.9U/mL] vs. median 2.4U/mL [IQR 1.9-3.0U/mL], p=0.464) and lupus anticoagulant (median 31.0 sec [IQR 29.7-34.6 sec] vs. median 31.4 sec [IQR 29.7-34.1 sec], p=0.998) showed no statistically significant differences between both groups.

No differences of leukocytes (median $5.78 \cdot 10^9$/L [IQR $4.95\text{-}6.39 \cdot 10^9$/L] vs. median $5.33 \cdot 10^9$/L [IQR $4.81\text{-}7.86 \cdot 10^9$/L], p=0.994), ESR (median 9mm/h [IQR 5-13mm/h] vs. median 8mm/h [IQR 4-13mm/h], p=0.450), CRP (median 1.3mg/L [IQR 0.6-2.1mg/L] vs. median 1.0mg/L [IQR 0.7-5.4mg/L], p=0.307), elevated ANA (95.4% vs. 93.8%, p=0.974), and ENA (median 21.5U/mL [IQR 14.0-31.0U/mL] vs. median 17.0U/mL [IQR 0.2-29.0U/mL], p=0.477) were found between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years. Furthermore, cardiolipin antibodies (median 1.4U/mL [IQR 0.9-2.0U/mL] vs. median 1.2U/mL [IQR 1.0-1.8U/mL], p=0.821), beta-2 glycoprotein antibodies (median 2.3U/mL [IQR 2.1-3.1U/mL] vs. median 2.6U/mL [IQR 2.2-2.9U/mL], p=0.775) and lupus anticoagulant (median 31.5 sec [IQR 29.7-35.8 sec] vs. median 30.8 sec [IQR 29.6-32.9 sec], p=0.198) did not differ between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years.

12.3 Correlation between endothelial dysfunction and inflammation

Significant positive correlations were found between FMD and NMD (r=0.541, p<0.001), between ADMA and SDMA (r=0.649, p<0.001) and between ADMA and CD31+/CD42b- EMP (r=0.236, p=0.040). Borderline significant positive correlation was also observed for CD31+/CD42b- EMP and SDMA (r=0.217, p=0.060) and borderline significant negative correlation between CD31+/CD42b- EMP and homoarginine (r=-0.221, p=0.055). Additionally, significant negative correlation was observed between FMD and aPWV (r-0.411, p<0.001). No further correlations were observed between FMD, NMD, aPWV, EMP, arginine, homoarginine, ADMA, and SDMA (Table 4).

As there were substantial differences between the group of lcSSc and controls regarding the correlations of endothelial dysfunction and inflammation, cumulative results of both groups of the respective correlations were not conducted. Instead, only correlations of the group of lcSSc regarding endothelial dysfunction and inflammation are given. Positive correlations were observed between ESR and aPWV (r=0.453, p=0.004) as well as between beta-2 glycoprotein antibodies and arginine (r=0.456, p=0.004). Negative correlations were observed between ESR and homoarginine (r=-0.324, p= 0.048), CRP and NMD (r=-0.430, p=0.012) as well as between cardiolipin antibodies and CD31+/CD42b- EMP (r=-0.325, p=0.047). No further correlations were observed between parameters of endothelial dysfunction and inflammatory parameters (Table 5).

		ADMA	SDMA	Arginine	Homoarginine	EMP CD31+/CD42-	aPWV	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				
EMP CD31+/CD42-	r	.236	.217	-.133	.107			
	p	.040	.060	.251	.358			
	n	76	76	76	76			
aPWV	r	.027	.066	-.014	-.221	.132		
	p	.815	.570	.903	.055	.255		
	n	76	76	76	76	76		
FMD	r	.089	.000	-.134	.195	.171	-.411	
	p	.443	.999	.248	.091	.140	.000	
	n	76	76	76	76	76	76	
NMD	r	-.085	-.212	.071	.113	.034	-.210	.541
	p	.498	.088	.570	.368	.784	.091	.000
	n	66	66	66	66	66	66	66

Table 4: Correlation matrix between parameters of endothelial dysfunction. Table reproduced from [237] with permission of Clinical and Experimental Rheumatology.

		FMD	NMD	aPWV	EMP CD31+/CD42-	Arginine	Homoarginine	ADMA	SDMA
Leukocytes	r	-.200	.077	.319	.081	.002	.065	.119	.087
	p	.228	.669	.051	.630	.991	.700	.475	.604
	n	38	33	38	38	38	38	38	38
ESR	r	-.120	-.120	.453	.130	.115	-.324	.308	.189
	p	.473	.505	.004	.438	.490	.048	.060	.256
	n	38	33	38	38	38	38	38	38
CRP	r	-.098	-.430	.212	-.013	.147	-.097	.188	.018
	p	.559	.012	.201	.937	.378	.564	.259	.913
	n	38	33	38	38	38	38	38	38
Positive ANA	r	-.014	-.205	.233	.221	-.120	-.139	.231	.085
	p	.931	.252	.160	.183	.475	.405	.163	.610
	n	38	33	38	38	38	38	38	38
ENA	r	-.146	.321	.275	.008	-.063	-.127	.034	.093
	p	.381	.068	.094	.960	.709	.447	.837	.580
	n	38	33	38	38	38	38	38	38
Cardiolipin antibodies	r	-.195	-.261	.076	-.325	.296	-.259	.183	.037
	p	.241	.142	.650	.047	.071	.116	.270	.825
	n	38	33	38	38	38	38	38	38
β2 glycoprotein antibodies	r	-.219	-.121	.016	-.191	.456	-.162	.306	-.008
	p	.186	.502	.922	.250	.004	.330	.062	.961
	n	38	33	38	38	38	38	38	38
Lupus anticoagulant	r	-.148	-.255	.072	-.213	-.006	-.112	-.009	.152
	p	.395	.174	.683	.220	.973	.524	.959	.383
	n	35	30	35	35	35	35	35	35

Table 5: Correlation matrix between parameters of endothelial dysfunction and inflammation. Table reproduced from [237] and adapted with permission of Clinical and Experimental Rheumatology.

12.4 Vascular events between lcSSc and controls

No difference between patients with lcSSc and controls were observed regarding feeling of xerostomia or xerophthalmia. Regarding specific parameters for PAH, higher DETECT score was present ($p<0.001$) in patients with lcSSc but no other PAH-specific parameter achieved statistically significant differences. Regarding skin involvement, patients with lcSSc have a higher mRSS than controls ($p<0.001$) and a higher frequency of skin changes, including telangiectasia, puffy finger and sclerodactyly, was observed in patients with lcSSc ($p<0.001$,

p<0.001, p<0.001, respectively). Lower values of eGFR and simultaneously higher values of creatinine were found in patients with lcSSc (p=0.008, p=0.042; respectively) while the remaining renal parameters did not differ between both groups. No adequate statistical analysis for urine immunoglobulin G and β2-microglobulin/creatinine ratio was conducted as the number of cases for those parameters between both groups was too low. No significant differences were observed for the total score of the UCLA SCTC GIT 2.0 questionnaire and also not for its subparameters. Impaired acral perfusion and the number of affected fingers with an impaired acral perfusion did not differ between both groups. Patients with lcSSc had more frequently an active and late pattern (p=0.002, p<0.001; respectively) in NVC, while the rate of an early pattern achieved borderline significance (p=0.054). A higher rate of capillaroscopic changes, including giant capillaries, capillary ramifications, bushy capillaries, microhemorrhages, capillary edema, capillary loss and disorganization of microvascular array, were observed in the group of lcSSC and those patients had also a higher CSURI. Detailed information about vascular events are listed in table 6.

Vascular event	LcSSc	Controls	p-value
Feeling of xerostomia/xerophthalmia, n (%)	24 (63.2)	22 (57.9)	0.815
Signs of PAH			
Dyspnea, n (%)	14 (36.8)	12 (31.6)	0.809
Right axis deviation, n (%)	0 (0)	0 (0)	-
P pulmonale, n (%)	0 (0)	0 (0)	-
Right branch block, n (%)	5 (13.2)	2 (5.3)	0.430
QTc prolongation > 450ms, n (%)	1 (2.6)	2 (5.3)	>0.999
Basal right ventricle diameter >42mm, n (%)	1 (2.6)	1 (2.6)	>0.999
Inferior caval vein diameter >21mm, n (%)	9 (23.7)	4 (10.5)	0.222
Estimated right atrial pressure >15mmHg, n (%)	1 (2.6)	1 (2.6)	>0.999
RV/LV ratio >1.0, n (%)	5 (13.2)	2 (5.3)	0.430
TAPSE <20mm, n (%)	7 (18.4)	3 (7.9)	0.309
Tricuspid regurgitation velocity >2.9m/s, n (%)	0 (0)	0 (0)	-
Tricuspid regurgitation velocity (m/s), median (25-75th percentile)	0.87 (0.58-1.13)	0.81 (0.47-1.13)	0.896
NT-proBNP > 150pg/ml, n (%)	9 (23.7)	6 (15.8)	0.565
Predicted single breath DLCO < 60%, n (%)	1 (2.6)	0 (0)	>0.999
DETECT step 1 (points), median (25-75th percentile)	309 (298-319)	292 (289-299)	**<0.001**
DETECT step 2 (points), median (25-75th percentile)	29 (24-33)	23 (19-25)	**<0.001**

Skin involvement			
mRSS (points), median (25-75th percentile)	4 (2-7)	0 (0-1)	**<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)	-
Renal involvement, median (25-75th percentile)			
eGFR (mL/min/1.73m²)	78.4 (71.6-90.9)	88.9 (76.7-95.8)	**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)	-*	-*	-*
Gastrointestinal involvement, median (25-75th percentile)			
UCLA SCTC GIT 2.0 reflux, (points)	0.13 (0.00-0.35)	0.00 (0.00-0.25)	0.150
UCLA SCTC GIT 2.0 distension, (points)	0.50 (0.00-1.25)	0.50 (0.00-1.25)	0.652
UCLA SCTC GIT 2.0 fecal soilage, (points)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.551
UCLA SCTC GIT 2.0 diarrhea, (points)	0.00 (0.00-0.50)	0.00 (0.00-0.00)	0.207
UCLA SCTC GIT 2.0 social functioning, (points)	0.00 (0.00-0.33)	0.00 (0.00-0.33)	0.476
UCLA SCTC GIT 2.0 emotional well-being, (points)	0.00 (0.00-0.11)	0.00 (0.00-0.22)	0.266
UCLA SCTC GIT 2.0 constipation, (points)	0.00 (0.00-0.50)	0.00 (0.00-0.50)	0.564
UCLA SCTC GIT 2.0 total, (points)	0.18 (0.06-0.41)	0.16 (0.09-0.30)	0.639
Microvascular involvement			
Impaired acral perfusion, n (%)	37 (97.4)	36 (94.7)	>0.999
Affected fingers with impaired perfusion, n	7 (4-10)	5 (4-8)	0.148
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**
Microhemorrhages, n (%)	23 (60.5)	3 (7.9)	**<0.001**
Capillary ramifications, n (%)	14 (36.8)	0 (0)	**<0.001**
Capillary loss, n (%)	9 (23.7)	0 (0)	**0.002**
Capillary edema, n (%)	8 (21.1)	0 (0)	**0.005**
Disorganization of microvascular array, n (%)	37 (97.4)	24 (63.2)	**<0.001**
Bushy capillaries, n (%)	14 (36.8)	5 (13.2)	**0.032**
CSURI (points), median (25-75th percentile)	0.0 (0.0-4.1)	-§	**<0.001**

Table 6: Bivariate analysis of vascular events between patients with lcSSc and controls. Table reproduced from [237] and adapted with permission of Clinical and Experimental Rheumatology.

*: The number of cases between both groups was too low for adequate statistical analysis.

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

In a subgroup analysis comparing lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years, those patients with a disease duration of > 7 years had a significant higher rate of clinical sclerodactyly than patients with a disease duration of ≤ 7 years (p=0.020). Additionally, higher rates of a present right branch block and a higher tricuspid regurgitation velocity were observed for the subgroup of lcSSc patients with a disease duration of ≤ 7 years although only borderline significance was achieved for the respective parameters (p=0.061, p=0.055; respectively). None of the remaining vascular events or disease-specific parameters differ between those subgroups. Detailed information about vascular events between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years are listed in table 7.

Vascular event	LcSSc ≤ 7 years	LcSSc > 7 years	p-value
Feeling of xerostomia/xerophthalmia, n (%)	14 (63.6)	10 (62.5)	>0.999
Signs of PAH			
Dyspnea, n (%)	8 (36.4)	6 (37.5)	>0.999
Right axis deviation, n (%)	0 (0)	0 (0)	-
P pulmonale, n (%)	0 (0)	0 (0)	-
Right branch block, n (%)	5 (22.7)	0 (0)	0.061
QTc prolongation > 450ms, n (%)	0 (0)	1 (6.3)	0.421
Basal right ventricle diameter >42mm, n (%)	1 (4.5)	0 (0)	>0.999
Inferior caval vein diameter >21mm, n (%)	7 (31.8)	2 (12.5)	0.254
Estimated right atrial pressure >15mmHg, n (%)	1 (4.5)	0 (0)	>0.999
RV/LV ratio >1.0, n (%)	4 (18.2)	1 (6.3)	0.374
TAPSE <20mm, n (%)	3 (13.6)	4 (25.0)	0.425
Tricuspid regurgitation velocity >2.9m/s, n (%)	0 (0)	0 (0)	-
Tricuspid regurgitation velocity (m/s), median (25-75th percentile)	0.95 (0.63-1.22)	0.72 (0.48-0.93)	0.055
NT-proBNP > 150pg/ml, n (%)	5 (22.7)	4 (25.0)	>0.999
Predicted single breath DLCO < 60%, n (%)	0 (0)	1 (11.1)	0.429
DETECT step 1 (points), median (25-75th percentile)	308 (298-313)	316 (294-328)	0.583
DETECT step 2 (points), median (25-75th percentile)	29 (26-32)	30 (23-36)	0.809

Skin involvement			
mRSS (points), median (25-75th percentile)	4 (2-7)	5 (3-9)	0.228
Telangiectasia, n (%)	4 (18.2)	7 (43.8)	0.147
Calcinosis cutis, n (%)	0 (0)	1 (6.3)	0.421
Puffy finger, n (%)	9 (40.9)	6 37.5)	>0.999
Sclerodactyly, n (%)	9 (40.9)	13 (81.3)	**0.020**
Acral necrosis, n (%)	0 (0)	0 (0)	-
Renal involvement, median (25-75th percentile)			
eGFR (mL/min/1.73m²)	82.0 (72.8-89.2)	76.5 (61.0-95.6)	0.654
Creatinine (mg/dL)	0.86 (0.75-0.92)	0.83 (0.70-0.98)	0.787
Protein/creatinine ratio (mg/g of creatinine)	100 (70-111)	93 (83-104)	0.753
Urine immunoglobulin G, mg/L	-*	-*	-*
Albumin/creatinine ratio (mg/g of creatinine)	13 (7-21)	12 (7-16)	0.792
α1-microglobulin/creatinine ratio (mg/g of creatinine)	11 (6-20)	10 (9-12)	0.787
β2-microglobulin/creatinine ratio (mg/g of creatinine)	-*	-*	-*
Gastrointestinal involvement, median (25-75th percentile)			
UCLA SCTC GIT 2.0 reflux, (points)	0.06 (0.00-0.35)	0.19 (0.00-0.35)	0.547
UCLA SCTC GIT 2.0 distension, (points)	0.75 (0.00-1.25)	0.13 (0.00-0.63)	0.116
UCLA SCTC GIT 2.0 fecal soilage, (points)	0.00 (0.00-0.00)	0.00 (0.00-0.00)	0.708
UCLA SCTC GIT 2.0 diarrhea, (points)	0.00 (0.00-0.50)	0.00 (0.00-0.00)	0.452
UCLA SCTC GIT 2.0 social functioning, (points)	0.08 (0.00-0.33)	0.00 (0.00-0.25)	0.605
UCLA SCTC GIT 2.0 emotional well-being, (points)	0.00 (0.00-0.11)	0.00 (0.00-0.06)	0.813
UCLA SCTC GIT 2.0 constipation, (points)	0.13 (0.00-0.50)	0.00 (0.00-0.50)	0.660
UCLA SCTC GIT 2.0 total, (points)	0.24 (0.06-0.41)	0.12 (0.05-0.41)	0.468
Microvascular involvement			
Impaired acral perfusion, n (%)	22 (100)	15 (93.8)	0.421
Affected fingers with impaired perfusion, n	6 (4-8)	8 (5-10)	0.419
Early pattern, n (%)	4 (18.2)	1 (6.3)	0.374
Active pattern, n (%)	4 (18.2)	5 (31.3)	0.450
Late pattern, n (%)	9 (40.9)	2 (12.5)	0.078
Giant capillaries, n (%)	8 (36.4)	6 (37.5)	>0.999
Microhemorrhages, n (%)	14 (63.6)	9 (56.3)	0.743
Capillary ramifications, n (%)	9 (40.9)	5 (31.3)	0.735
Capillary loss, n (%)	6 (27.3)	3 (18.8)	0.706
Capillary edema, n (%)	6 (27.3)	2 (12.5)	0.426
Disorganization of microvascular array, n (%)	22 (100)	15 (93.8)	0.421
Bushy capillaries, n (%)	8 (36.4)	6 (37.5)	>0.999
CSURI (points), median (25-75th percentile)	0.0 (0.0-4.1)	0.0 (0.0-3.7)	0.929

Table 7: Bivariate analysis of vascular events between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years.

*: The number of cases between both groups was too low for adequate statistical analysis.

12.5 Periodontal inflammation between lcSSc and controls

Patients with lcSSc had more affected gums than controls on BOP testing (55.6% vs. 42.4%), which was however not significant (p=0.116). A pocket depth >3.5mm indicating periodontal attachment loss was present in 97.3% of patients with lcSSc and in 97.4% of patients with primary Raynaud's phenomenon (p=0.985). There was no significant difference between lcSSc patients with a disease duration of ≤ 7 years and lcSSc patients with a disease duration of > 7 years regarding BOP (58.3% vs. 51.8%, p=0.592) and a pocket depth >3.5mm (95.5% vs. 100%, p=0.417).

12.6 Correlation between endothelial dysfunction and vascular events

Correlation analyses were performed between endothelial dysfunction and selected vascular events which achieved in bivariate analysis statistical significance (DETECT score step 1 and step 2, mRSS, eGFR, early, active and late pattern, and CSURI). As puffy fingers and sclerodactyly are reflected by the mRSS, creatinine is reflected by eGFR, as well as telangiectasia and morphological capillary changes are reflected by early, active, and late pattern, those parameters are not included in correlation analysis. Furthermore, as parameters of periodontal inflammation did not achieve significant differences, no correlation analysis was conducted for these respective parameters.

Due to substantial differences between the group of lcSSc and controls regarding the correlations of endothelial dysfunction and vascular events, only correlations of the lcSSc group of lcSSc are presented. Significant correlation was observed between DETECT score step 1 and SDMA (r=0.376, p=0.020) and borderline significance was found between DETECT score step 1 and ADMA (r=0.307, p=0.061). DETECT score step 2 correlated also positively with ADMA (r=0.328, p=0.045) and with SDMA (r=0.328, p=0.045). SDMA and aPWV revealed negative correlations with eGFR (r=-0.560, p<0.001; r=-0.424, p=0.008; respectively).

Additionally, CSURI correlated with aPWV (r=0.370, p=0.022), CD31+/CD42b- EMP (r=-0.327, p=0.045) and ADMA (r=0.329, p=0.043). No further correlations were observed between parameters of endothelial dysfunction and vascular events (Table 8).

Multivariate analysis was not possible due to a too low number of subjects with lcSSc.

		FMD	NMD	aPWV	EMP CD31+/CD42	Arginine	Homoarginine	ADMA	SDMA
mRSS	r	-.176	-.160	.223	-.211	-.103	-.146	-.103	-.214
	p	.292	.373	.179	.204	.539	.382	.539	.198
	n	38	33	38	38	38	38	38	38
DETECT score step 1	r	.007	-.108	.202	.098	.199	-.181	.307	.376
	p	.966	.551	.225	.560	.231	.277	.061	.020
	n	38	33	38	38	38	38	38	38
DETECT score step 2	r	.050	-.144	.176	.178	.089	-.069	.328	.426
	p	.768	.424	.291	.285	.596	.681	.045	.008
	n	38	33	38	38	38	38	38	38
eGFR	r	.085	.118	-.424	.040	-.045	.160	-.233	-.560
	p	.614	.512	.008	.813	.787	.338	.159	<.001
	n	38	33	38	38	38	38	38	38
CSURI	r	-.001	-.007	.370	.327	-.058	-.002	.329	.188
	p	.994	.970	.022	.045	.728	.990	.043	.258
	n	38	33	38	38	38	38	38	38
Early pattern	r	-.032	-.059	.039	-.078	.039	.185	-.210	-.018
	p	.860	.770	.826	.625	.834	.275	.213	.925
	n	38	33	38	38	38	38	38	38
Active pattern	r	-.042	.007	-.280	-.240	-.025	-.229	-.234	-.178
	p	.807	.984	.091	.149	.893	.170	.159	.288
	n	38	33	38	38	38	38	38	38
Late pattern	r	.050	-.201	.196	.214	.151	-.050	.249	.106
	p	.769	.269	.240	.198	.373	.769	.134	.530
	n	38	33	38	38	38	38	38	38

Table 8: Correlation matrix between parameters of endothelial dysfunction and vascular events. Table reproduced from [237] and adapted with permission of Clinical and Experimental Rheumatology.

13. Discussion

This thesis could demonstrate that selected parameters of endothelial dysfunction differ in patients with lcSSc compared to age-, race- and sex-matched controls with primary Raynaud's phenomenon suggesting that mechanisms contributing to SSc-related vasculopathy and vascular changes vary distinctively between each other.

FMD and NMD are parameters of peripheral vascular reactivity. Both are mediated by several vasoactive agents, including NO, prostaglandins, adenosine and myogenic relaxation, while also other factors, like age, sex, and cardiovascular risk factors, may influence FMD and NMD [169,171,176,179,255,256]. In this thesis, neither FMD nor NMD differ between both groups while a correlation between both parameters was observed. These findings are consistent with data of previous studies. Although a meta-analysis reported that FMD and NMD are lower in patients with SSc, individual studies reported wide variations of measured FMD and NMD ranging from no differences to distinctively impaired values [196,257-262]. This discrepancy is primarily caused due to heterogenous investigation methods. Several studies did not compare FMD or NMD values with a control cohort or, if a control group was used, those controls were commonly unmatched to age or sex [182,184,232,260]. Additionally, the investigated cohorts of patients with SSc were heterogenous regarding different SSc subtypes [180,181,257,258]. Moreover, several studies investigating FMD and NMD included patients with different clinical manifestations and symptoms of SSc, like digital ulcers or PAH, and comorbidities like atherosclerotic diseases [232,263,264]. All of them may additionally have influenced the reported results of reported FMD in SSc. Domsic et al. [262] reported similar investigation methods like this study and found comparable results that endothelial dysfunction assessed by FMD, NMD and also aPWV did not significantly differ between patients with SSc and controls. As patients with primary Raynaud's phenomenon did not exhibit pathologic values of FMD and NMD, the use of those patients as controls was appropriate [265,266]. Regarding absolute values of FMD and NMD, this thesis found similar results compared to prior studies [257,258,262]. Compared to proposed reference values of FMD and NMD, both groups revealed diminished FMD values, while NMD values were within the normal range. This suggests that endothelial-independent vasodilation may be intact in lcSSc while a reduced NO synthesis with reduced arterial capacity and vasoconstriction prevail. Potential contributors of an impaired NO synthesis are ADMA and SDMA, which were significantly elevated in patients with lcSSc [214,219]. Furthermore, ADMA acts also as a vasoconstrictive agent [267]. However, the additional impact of ADMA and SDMA on FMD, besides the influence of age, sex and several cardiovascular risk factors, may be marginal as neither ADMA nor SDMA

correlated with FMD. This hypothesis may be supported by the reason why FMD did not differ to the control group. Patients with primary Raynaud's phenomenon were age- and sex-matched, and have also a comparable distribution of cardiovascular risk factors. As neither arginine, nor homoarginine, nor CD31+/CD42b- EMP, nor inflammation parameters correlated with FMD, this suggests, on the one hand, that other mediators may contribute to a reduced NO synthesis, or, on the other hand, that FMD is altered by many different mediators with a variable magnitude.

APWV is a parameter of arterial stiffness which is also mediated by age, sex and cardiovascular risk factors [268-270]. Increased aPWV is an independent marker of cardiovascular mortality and a parameter of macrovascular endothelial dysfunction [185,192]. Comparably to FMD and NMD, aPWV did not differ in this study between patients with lcSSc and controls. Both groups revealed aPWV values within the reported range although there was a significant negative correlation with FMD. Previous studies reported similar results with absent differences of aPWV between SSc and controls [259,271]. Additionally, comparable correlation of aPWV to FMD were also observed in other autoimmune diseases and in healthy adults [272,273]. This finding suggests that processes affecting the arterial wall promote to a similar extent arterial stiffness and pathologic vascular reactivity. Although a borderline significantly, negative correlation between aPWV and homoarginine was also observed, this finding seems to be rather incidental as no other parameter of the arginine metabolism correlated in a comparable degree with aPWV. Comparably, only ESR correlated positively with aPWV but no further inflammatory parameter, suggesting that this finding was incidental. Therefore, a potential promotion of the arginine metabolism on arterial stiffening may be present, like in FMD, but seems to be marginal.

EMP are relatively new identified parameters of endothelial dysfunction which are released by activation and apoptosis of endothelial cells. Different EMP phenotypes can be identified by specific cell-surface markers indicating the origin of the respective EMP phenotype and those EMP phenotypes contribute to endothelial changes, including endothelial inflammation, angiogenesis, coagulation, and structural endothelial modifications [198,202-206]. Interestingly in this thesis, only CD31+/CD42b- EMP could be detected in both groups with borderline significantly higher values in the group of lcSSc, while CD51+/CD42b-, CD54+/CD42b-, CD62E+/CD42b-, CD105+/CD42b-, and CD144+/CD42b- EMP were undetectable by the above described method. This finding may be related primarily to technical and physiological factors. The measurement method of EMP is still under investigation without present gold standard and differs between previously published data on SSc [208-

211,274,275]. In our protocol, we essentially followed the recommendations for the analysis of extracellular vesicles by flow cytometry as published by Cossarizza et al. [276]. Our measurement method differs primarily in two ways from previous studies. Firstly, we used silica particles for size calibration of the flow cytometer in contrast to polystyrene particles. It was shown that the refractive index of silica particles is much closer to the refractive index of extracellular vesicles. Therefore, they are superior to polystyrene particles for estimation of the size of extracellular vesicles and hence to the definition of the extracellular vesicle gate on scatter plots. The absence of CD markers other than CD31 in our study could be the result of different size calibration. Secondly, we used fluorescence triggering on lactadherin fluorescence for the detection of EMP. Lactadherin specifically binds to phosphatidylserine and thus, only EMP expressing phosphatidylserine are detected by our approach while phosphatidylserine negative EMP expressing other reported markers would be missed in our analysis. Additionally, EMP generation can be very easily falsified by various technical and physiological influencing factors, including hemolysis, venous stasis, age or menstrual cycle [207,277]. Furthermore, although slight hemolysis was present in only one sample and venous stasis as well as shaking of the blood sample were avoided in each blood sample, it may be possible that other, yet unknown factors had influenced the quality of the samples significantly so that other EMP phenotypes were undetectable. Moreover, as some of the respective EMP phenotypes were associated so far only in SSc patients with specific vascular complications, like PAH, it may be possible that PAH-naïve lcSSc patients and those free from digital ulcers do not release specific EMP phenotypes. Potential interacting pathways between EMP and NO or arginine metabolism have not been described yet while this thesis could demonstrate a significant correlation between CD31+/CD42b- EMP and ADMA and also a borderline significant correlation with SDMA. Microparticles represent a heterogenic group as they may derived from several different cell types featuring numerous antigens [198,277]. Due to this heterogeneity, microparticles exhibit also different functional effects and they may also affect cells different from those from which they have been initially derived. T-cell-derived microparticles may induce a decrease of eNOS by regulation of its protein expression promoting thus endothelial dysfunction [278]. Additionally, other studies reported inhibiting effects of microparticles and EMP on eNOS [279,280]. Although data on CD31+/CD42b- EMP affecting NO metabolism are yet lacking, the positive correlations between CD31+/CD42b- EMP, ADMA and SDMA suggest a potential influence. However, although endothelial cell activation and damage reflected by EMP might be present in patients with lcSSc, as also suggested by previous studies, those results remain yet controversial due to inhomogeneous patient cohorts and measurement methods [207-211,274,275]. An establishment of a validated

measurement method of EMP and investigations of larger patient cohorts with different SSc subtypes and SSc-related complications are urgently needed to clarify the significance of EMP in this disease.

Arginine, homoarginine, ADMA and SDMA represent important mediators of the NO metabolism and are relevant predictors of cardiovascular diseases [220-227]. Although numerous data of these parameters are available in cardiovascular diseases, their role as mediators of endothelial dysfunction in lcSSc is much less investigated yet. So far, data about arginine and homoarginine as potential contributors of endothelial dysfunction in lcSSc and predictors of SSc-related complications are very limited. In this thesis, neither arginine nor homoarginine differ between patients with lcSSc and primary Raynaud's phenomenon. Furthermore, no correlation to other parameters of endothelial dysfunction was observed, except a borderline significantly positive correlation of homoarginine to aPWV, which was, however, rather a random finding. Contrary, ADMA and SDMA were significantly elevated in patients with lcSSc and revealed also a positive correlation. Elevated ADMA has been reported in previous studies on SSc contributing to SSc-related complications [230-232]. The fact, that SDMA is also elevated and correlated with ADMA, was yet unreported. As this study did not include patients with vasculopathy-associated SSc-related complications, it seems that ADMA and SDMA might promote endothelial dysfunction in lcSSc already at an early stage.

Inflammation parameters did not differ between both groups in this thesis and were not elevated, except for ANA and ENA with higher levels in lcSSc patients, which was however expectable as both parameters indicate pathologic autoimmunity in this autoimmunological disorder. Additionally, only occasional and divergent correlations were found in this thesis between parameters of endothelial dysfunction and inflammation parameters. Beta-2 glycoprotein antibodies correlated positively with arginine while cardiolipin antibodies correlated negatively with CD31+/CD42b- EMP suggesting that both antiphospholipid antibodies might have a protective effect on the endothelium which is contrary to previous data [281]. Negative correlations were observed between ESR and homoarginine and between CRP and NMD suggesting a potential promotion of endothelial dysfunction by inflammatory processes. However, this promotion may be marginal as only those respective parameters correlated with each other and inflammation was overall negligible as no inflammatory parameter was pathologically elevated. Similar results were also observed in previous studies suggesting that inflammatory parameters do not contribute to endothelial dysfunction or have only a marginal effect on it [282,283].

Vascular events were defined as vasculopathy-associated clinical changes, including potential cutaneous, renal, gastrointestinal and microvascular alterations as well as signs of PAH and sicca symptoms of mouth and eyes, as those changes may be triggered by vasculopathy [50,52,69,233-236]. Between patients with lcSSc and controls, a higher mRSS score as well as higher rates of SSc-related skin and capillary changes were observed, which was however expectable as lcSSc affects the skin and microvasculature and those changes are also part of the recent diagnostic criteria for SSc [1]. Interestingly, patients with lcSSc had a significantly higher DETECT score than controls although PAH was an exclusion criterion and no other specific parameter or sign indicating potential PAH differed between both groups. As the DETECT score represents a tool for PAH screening in lcSSc, this finding suggests that single clinical changes indicating PAH may be marginally apparent in PAH-naïve lcSSc whereas the sum of those changes might be clinically relevant. Similarly, patients with lcSSc had lower values of eGFR and higher values of creatinine although SSc-related renal involvement was known in only two patients and no other renal parameter differed between both groups, suggesting that subclinical nephropathy may be present in these patients. Comparable results had been described earlier [284]. No further vascular event differed between patients with lcSSc and controls. Sicca symptoms, including xerostomia and xerophthalmia, were only evaluated in this study by a questionnaire with self-assessment, but without objective measurements. As xerophthalmia and xerostomia are common among general population with an estimated prevalence of 15-20%, especially in females and older people, it might be influenced the results of this thesis [285,286]. The results of gastrointestinal involvement by the UCLA SCTC GIT 2.0 questionnaire and of periodontal inflammation might be also influenced by the subjective survey and by other risk factors. Although UCLA SCTC GIT 2.0 questionnaire is a validated tool in SSc reflecting gastrointestinal symptom severity and impact on quality of life, numerous other conditions and diseases, including irritable bowel syndrome or food intolerances, manifest with comparable symptoms and biasing thus results of this questionnaire. Periodontal inflammation is another common disease among general population which may be aggravated by several confounding factors, including tobacco abuse and arterial hypertension [287-289]. As there was a high prevalence of tobacco abuse and arterial hypertension also in the control group, this may bias also the results on periodontal inflammation.

ADMA and SDMA revealed significant correlations with the DETECT score. In previous studies, ADMA was correlated with present PAH in patients with SSc-related PAH while data about correlations of SDMA and PAH are missing [290,291]. These results suggest that selected parameters of the NO metabolism may be involved in the development of pulmonary

vascular disease already at an early stage. ADMA and CD31+/CD42b- EMP were also correlated with CSURI. It could be previously demonstrated that elevated levels of ADMA may be a predictor for the development of digital ulcers in patients with SSc [232]. Furthermore, CD31+/CD42b- EMP showed an inverse correlation with CSURI while no correlations were found for any capillary patterns. However, this finding suggests that CD31+/CD42b- EMP reflect rather the degree of endothelial apoptosis than endothelial activation because positive but not significant correlation to capillary late pattern was observed while not significantly, negative correlations to early and active pattern were achieved. This finding is also contrary to previous results presented by Michalska-Jakubus et al. [209] in which negative correlations to capillary changes of late pattern were found. Furthermore, CSURI is an index depending on the number of mega capillaries and total number of capillaries and therefore rather a parameter of endothelial activation than endothelial apoptosis. CSURI was also correlated with aPWV although aPWV values were higher in controls suggesting that this finding is rather a random finding. However, correlations between arterial stiffness and CSURI have been reported previously but also not with aPWV [292].

Within this thesis, SDMA revealed also a strong correlation to eGFR indicating early renal damage, similarly to previous studies as SDMA is mainly excreted by the kidneys and patients with end-stage renal disease having also the highest SDMA levels due to the impaired renal function [293,294]. Furthermore, aPWV correlated to renal function in this thesis which is also concordant to previous results about aPWV in patients with chronic kidney disease [295]. Further studies are needed if SDMA and aPWV may be associated with SSc-related nephropathy.

No differences of parameters of endothelial dysfunction or vascular events were observed within the group of patients with lcSSc and a disease duration of ≤ 7 years and patients with a disease duration of > 7 years. Only sclerodactyly occurred more often in lcSSc patients with a disease duration of > 7 years. It seems that changes contributing to endothelial dysfunction occur at a very early stage of lcSSc and do not deteriorate necessarily over time. Additionally, no vascular event or specific parameter, except sclerodactyly, occurred more frequently in the early or late stage of lcSSc. As Raynaud's phenomenon commonly precedes the diagnosis of lcSSc by several years, it is possible that also endothelial dysfunction is promoted at that initial phase and remains constantly afterwards [142].

One limitation of the present study is the relatively small sample size although this study included homogenous cohorts of lcSSc patients defined by the recent ACR/EULAR criteria and of age-, race- and sex-matched controls. Another limitation is the evaluation of sicca symptoms

and gastrointestinal involvement by subjective surveys. Objective measurements including ophthalmologic examination for xerophthalmia or Saxon test for hyposalivation would be more suitable. Additionally, long-term follow-up investigations clarifying if specific parameters of endothelial dysfunction are potential predictors for the development of SSc-related, vascular complications would be interesting.

In conclusion, selected parameters of endothelial dysfunction may contribute to vasculopathic changes in patients with lcSSc suggesting that endothelial dysfunction is primarily present in microvasculature. The impact on macrovascular changes including vascular reactivity and arterial stiffness remains still indistinctively in lcSSc. Positive correlations between CD31+/CD42b- EMP, ADMA and SDMA may indicate additionally potential interactions of endothelial activation and NO metabolism.

14. References

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