Micro- and macrovascular abnormalities in systemic sclerosis

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CHAPTER 1

GENERAL INTRODUCTION

CHAPTER 1

SYSTEMIC SCLEROSIS

Systemic sclerosis (scleroderma, SSc) is an autoimmune connective tissue disease of unknown etiology characterized by cutaneous and visceral fibrosis, and widespread vascular pathology. Preliminary criteria for the classification of systemic sclerosis have been proposed by the American College of Rheumatology (formerly, the American Rheumatism Association) in 1980 (table 1).[1] These proprosed criteria had a 97% sensitivity and 98% specificity for definite systemic sclerosis in the presence of the major criterion, or two or more of the minor criteria. The main and major diagnostic criterion for SSc is fibrosis of the skin, whereas fibrosis is also a major part of the minor criteria.

Table 1. Preliminary clinical criteria for systemic sclerosis[1]

Major criterion
Proximal scleroderma
Minor criteria
Sclerodactyly
Digital pitting scars or loss of substance of the distal finger pad
Bibasilar pulmonary fibrosis on standard chest X-rays

A classification of SSc patients into 2 subsets was proposed by LeRoy et al.[2] Limited cutaneous SSc (lcSSc) is defined by skin involvement limited to hands, face, feet, and forearms (acral). In diffuse cutaneous SSc (dcSSc) truncal and acral skin involvement is also present. Subsets differ also in the presence of autoantibodies and visceral disease (table 2).

Table 2. Subsets of systemic sclerosis[2]

Diffuse cutaneous SSc (dcSSc)
Onset of Raynaud's within 1 year of onset of skin changes (puffy or hidebound)
Truncal and acral skin involvement
Presence of tendon friction rubs
Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, and myocardial involvement
Absence of anticentromere antibodies (ACA)
Nailfold capillary dilation and capillary destruction
Antitopoisomerase antibodies (30% of patients)
Limited cutaneous SSc (lcSSc)
Raynaud's for years (occasionally decades)
Skin involvement limited to hands, face, feet, and forarms (acral) or absent
A significant incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia, skin calcifications, teleangiectasia
A high incidence of ACA (70-80%)
Dilated nailfold capillary loops, usually without capillary dropout

Skin thickening provides the definitive diagnostic criterion in SSc in over 90% of patients.[1] However, the presence of vascular disease, such as Raynaud's phenomenon, a major clinical manifestation in SSc patients, is not part of the classification criteria.

RAYNAUD'S PHENOMENON AND VASCULAR INVOLVEMENT

Raynaud's phenomenon

Raynaud's phenomenon (RP) is characterized by episodic digital vasospasm provoked by cold and/or emotional stress. Without evidence of an associated disorder RP is considered primary. With evidence of an associated disorder, like SSc, RP is considered secondary.
A typical episode or attack is triphasic. First, vasospasm occurs with sharply demarcated white color changes of skin, then blue discoloration occurs (cyanosis), followed after rewarming by vasodilatation with erythema of reperfusion (red). In some patients only the white or cyanotic phase is seen, both always followed by the red phase. The signs of uncomplicated RP should be completely reversible after rewarming or reduction of stress. In severe secondary RP, pain or ulceration of the skin (typically the tips of the fingers and toes) may result from critical tissue ischemia.[3;4] In patients diagnosed as primary RP, 7 % to 13% eventually develop a secondary disorder, most frequently SSc, mixed connective tissue disease (MCTD), systemic lupus erythematosus (SLE) or Sjögren's syndrome.[5-7] Otherwise, in patients with a connective tissue disease RP is a common manifestation. For example, RP is present in more than 95% of patients with SSc. Its presence together with other factors like the frequency and the severity of the attacks and the presence of digital ulcers affects the quality of life of these patients.[8] The pathogenesis of RP is not yet fully understood, but is considered multifactorial. There is evidence that RP attacks are due to a local fault at the level of the digital microcirculation, in which digital arteries are hypersensitive to cold. Lewis observed in the first decades of the 20th century that vasospastic attacks can still occur in RP patients after blockade of sympathic nerves, such as after local anesthesia of the digital sympathetic nerves, or after sympathectomy, and suggested that a functional abnormality is responsible.[9;10] Otherwise, evidence is present revealing an increased expression or activity of postjunctional α_2 adrenoreceptors.[11-16] Other, non-adrenergic mechanisms play an important role in the pathogenesis. Disturbances in the regulation of vascular tone with decreased or inadequate vasodilatory signals and increased or enhanced vasoconstrictive signals can result in RP,[16;17] as in healthy state vascular tone is controlled by a delicate interplay between these factors.

Endothelial dysfunction

Campbell and LeRoy proposed their vascular hypothesis in 1975 considering the vascular abnormalities and the presence of RP prior to scleroderma skin manifestations.[18] Hereby, vascular abnormalities and dysfunction are considered to be an important element in SSc. Vascular abnormalities are noted in the capillaries and small blood vessels. Using nailfold microscopy or digitised video capillaroscopy, characteristic nailfold capillary abnormalities are found, like decreased capillary density, enlarged and giant capillaries, haemorrhages, disorganization of the vascular array and ramified/bushy capillaries.[19-23] Besides structural abnormalities, vascular dysfunction plays an important role. The endothelial cell is responsible for vascular permeability, control of vascular tone, control of haemostasis and thrombosis, and can interact with leucocytes.[24] Circulating markers of endothelial cell function, such as von Willebrand factor (vWF), tissue plasminogen activator, soluble E-selectin (sE-selectin), soluble vascular cell adhesion molecule 1 (s-VCAM-1), soluble intercellular adhesion molecule 1 (s-ICAM-1), and endothelin, are raised in a significant

proportion of SSc patients.[25-30] Also, the level of circulating endothelial cells (CECs), proposed to be a reliable marker of endothelial damage in different vascular diseases, is elevated in SSc, and related to disease activity and the presence of pulmonary hypertension.[31] Increased capillary permeability of the nailfold capillaries has been found by using dynamic fluorescence videomicroscopy of the nailfold.[32] Studying vascular tone, impaired endothelium-dependent vasodilation has been found in SSc using laser Doppler fluxmetry in combination with iontophoresis or other dynamic tests, like cooling and post-occlusive hyperemia.[33-36] Laser Doppler fluxmetry is well established in the measurement of cutaneous microcirculatory flow. Other non-invasive methods for evaluating cutaneous microcirculatory flow have been described recently by Wright et al.[37] To study endothelial function in conduit arteries, ultrasound has been used. Using brachial artery ultrasound-derived flow-mediated dilation (FMD), impaired endothelial-dependent vasodilation has also been found. [38-40] Endothelial dysfunction is strongly implicated in the pathogenesis of atherosclerosis.[41;42] An increased prevalence of atherosclerosis has been found in other auto-immune diseases, such as systemic lupus erythematosus, in which endothelial dysfunction is present.[43-45] In SSc, vascular involvement has always been considered to be mainly microvascular,[46] but in the last two decades macrovascular disease has also been described.[47-49]

Endothelin-1

Endothelin is a potent vasoconstrictor peptide originally characterized from the culture supernatant of porcine aortic endothelial cells.[50] The precursor of endothelin is a 212-residue prepropeptide cleaved in two steps into the active 21-amino acid endothelin. Three structurally and pharmacologically distinct isopeptides are present, produced by three seperate genes.[51] Endothelin-1 (ET-1) is the most significant isoform. Endothelin-2 (ET-2) is produced predominantly within the kidney and intestine, but has no specific physiological function. Endothelin-3 (ET-3) circulates in plasma, and has been found in the brain, gastrointestinal tract, lung and kidney. ET-3 is probably involved in the central nervous system. Endothelin-1 (ET-1) is produced in endothelial cells, but also in vascular smooth muscle cells. The plasma half-life is short and is approximately 4 to 7 minutes. As such, vascular tone can be rapidly adjusted depending on ET-1 concentrations. ET-1 binds to specific receptors on smooth muscle cells causing vasoconstriction.[52] There are two endothelin receptors, ET_A and ET_B. The ET_B receptor binds ET-1, ET-2 and ET-3 equally, while the ET_A receptor has a strongly preferential binding affinity for ET-1 above ET-3.[53] Increased plasma ET-1 levels have been found in patients with RP, primary and secondary, and in patients with SSc[54-60], suggesting that ET-1 plays a part in the pathogenesis of these conditions. However, others found no differences in ET-1 levels in plasma in resting conditions and after cold provocation in patients with primary RP and/or secondary RP compared with healthy controls.[61-63] ET-1 may also be important in the pathogenesis of the fibrotic manifestations of SSc. ET-1 is profibrogenic by enhancing fibroblast proliferation and collagen synthesis,[57] and ET-1 levels are found to correlate with several disease characteristics, amongst others skin fibrosis.[64] ET-1 was shown to induce a fibrogenic phenotype in normal fibroblasts similar to that of lesional SSc fibroblasts.[65] Since ET-1 plays an important role in vascular and fibrotic lesions in SSc, use of specific endothelin-receptor antagonists may be a promising therapy for both vascular and fibrotic lesions in

SSc. Bosentan is an orally active dual endothelin receptor antagonist, shown to be effective in the treatment of idiopathic pulmonary arterial hypertension and in SSc-related pulmonary hypertension.[66-70] Bosentan was also found to be effective in the prevention of new digital ulcers in SSc patients,[71] and case-series have suggested a positive effect of the drug on RP.[72-74]

OUTLINE OF THIS THESIS

This thesis describes several aspects of vascular involvement in SSc, i.e. RP, microvascular involvement, endothelial cell dysfunction, and macrovascular involvement.

Microvascular involvement and endothelial cell dysfunction
In SSc treatment with vasodilating agents, including calcium-channel blockers, angiotensin converting enzyme inhibitors, serotonin receptor blockers and intravenous prostaglandin analogues, are used for RP. However, these agents showed only moderate reduction in severity and frequency of attacks. Side-effects frequently result in discontinuation of these agents.
Endothelin-1 (ET-1) has been suggested to play a role in the pathogenesis of RP. We hypothesized that bosentan, an ET receptor antagonist, could be a useful agent in the treatment of RP. Furthermore, we questioned whether these effects could be explained by the effects of bosentan on endothelial cell dysfunction in SSc patients with severe RP.

In **chapter 2** the results of treatment with bosentan on frequency, duration and severity of RP attacks are described in patients with RP secondary to SSc. These effect were assessed by diary and, more objectively, by photoelectric plethysmography during cooling and rewarming.

Chapter 3 describes the effects of treatment with bosentan on endothelial cell dysfunction by assessment of vasodilatory microvascular reponses using laser Doppler fluxmetry combined with iontophoresis, capillary permeability using fluorescence videomicroscopy, nailfold capillary microscopy, and serological markers of endothelial activation.

Vascular or endothelial cell dysfunction has been suggested to be a crucial element in the pathogenesis of SSc. In **chapter 4** we describe microvascular reactivity in SSc patients and healthy controls. We evaluated endothelial microvascular function, assessed by laser Doppler flowmetry combined with iontophoresis, and determined whether endothelium-dependent microvascular reactivity was related to the presence of SSc or other possible confounding variables, such as hypertension, diabetes and obesity.

In **chapter 5** the results are presented of a case-control study in which we investigated capillary permeability in SSc patients and healthy controls. The primary objective of this study was to investigate whether increased capillary permeability in nailfold capillaries found in patients with SSc, is a generalized phenomenon. We used sodium fluorescence videodensitometry of the ankle to test this hypothesis.

Macrovascular involvement

Microvascular involvement is common in SSc and the frequency of vascular abnormalities was found to be inversely proportional to the size of blood vessels studied. However, less data are available on the prevalence of macrovascular involvement in SSc.

In **chapter 6** the literature on macrovascular involvement in SSc is reviewed.

In **chapter 7**, we describe the prevalence of early signs of atherosclerosis by measuring intima media thickness (IMT) of the common carotid artery in patients with SSc compared to healthy controls. Outcome is related to the presence of disease-related and traditional cardiovascular risk factors.

Accumulation of advanced glycation endproducts (AGEs) is related to age, but occurs more rapid in conditions like diabetes mellitus, renal failure, atherosclerosis and inflammatory diseases, like rheumatoid arthritis. Tissue autofluorescence is related to the accumulation of AGEs. In **chapter 8**, we assessed skin autofluorescence by using the Autofluorescence Reader in patients with SSc in comparison with healthy controls. We related the extent of AGE accumulation to the presence of disease-related and traditional cardiovascular risk factors.

Finally, the results and conclusions of this thesis are summarized in **chapter 9**.

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CHAPTER 2

BOSENTAN THERAPY FOR PATIENTS WITH SEVERE RAYNAUD'S PHENOMENON IN SYSTEMIC SCLEROSIS

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CHAPTER 2

ABSTRACT

Objective: Raynaud's phenomenon (RP) and the presence of digital ulcers affects the quality of life of patients with systemic sclerosis (SSc). Although the etiology of RP is multifactorial, the endothelium derived vasoconstrictor molecule endothelin-1 (ET-1) has been suggested to play a role in its pathogenesis. We evaluated the effects of bosentan on RP in patients with SSc with or without digital ulcers.
Methods: In this single center, observational study, 15 patients were treated with bosentan for 16 weeks with a follow-up period of 4 weeks. Outcome measures were the frequency, duration and severity of RP, recorded in a diary. Photoelectric plethysmography during cooling and rewarming was performed at baseline and at week 8, 16 and 20.
Results: At week 16, bosentan treatment resulted in a significant reduction in the total daily duration of RP attacks ($p=0.001$), the daily number of RP attacks ($p<0.001$) and severity of RP attacks, measured by the Raynaud's Condition Score (RCS) ($p=0.003$). Although the mean daily outdoor temperature significantly increased after 10 weeks of treatment, a significant reduction in RP outcome measurements was already observed at this point. After discontinuation of bosentan no significant changes in RP outcome measurements were seen during the 4-week follow-up period. Treatment was not associated with significant differences in flow during cooling and rewarming recorded by photoelectric plethysmography.
Conclusion: The endothelin-1 receptor antagonist bosentan resulted in a subjective improvement of the daily duration, number and severity of RP attacks in this open, uncontrolled study.

INTRODUCTION

Systemic sclerosis (SSc) is an autoimmune connective tissue disease characterized by fibrosis of the skin and visceral organs, and vascular abnormalities, including Raynaud's phenomenon.[1;2] Raynaud's phenomenon (RP), characterized by episodic digital vasospasm provoked by cold and/or emotional stress, is present in more than 95% of patients with SSc. Its frequency and severity in combination with the presence of digital ulcers affects the quality of life of patients.[3] Although treatment with different vasodilators including calcium-channel blockers, angiotensin converting enzyme inhibitors, serotonin receptor blockers and intravenous prostaglandin analogues are used in common practice, alternative treatments that are more effective or have a better side-effects profile are needed.
Although the etiology of RP is multifactorial, the endothelium derived vasoconstrictor molecule endothelin-1 (ET-1) has been suggested to play a role in its pathogenesis. Evidence, however, is conflicting as some authors showed no differences in ET-1 levels in plasma of primary RP (pRP) and/or secondary RP (sRP) patients when compared with healthy controls,[4-6] while others found a significant increase in RP patients.[7-13] Elevated ET-1 levels may be caused by a stressed endothelium[14] or simply be a marker of endothelial damage.[15] Studies have shown that other markers of endothelial damage are related to disease severity in patients with pRP and sRP.[16] Blocking of the ET-1 receptor, therefore, might be interesting. Therapeutically, the use of bosentan, an ET receptor antagonist, has been shown effective in patients with ischemic ulcers in SSc.[17] In view of the possible pathogenic role of ET-1 in RP and the efficacy in preventing development of new digital ulcers in patients with SSc, we hypothesized that bosentan can also be a useful and effective therapeutic strategy in patients with sRP.
This observational study was conducted to assess the effects of bosentan on Raynaud's phenomenon in patients with SSc with or without digital ulcers.

PATIENTS AND METHODS

Patients

Patients from the outpatient clinic with a diagnosis of RP secondary to SSc were eligible for the study. Patients had to be at least 18 years old, have a diagnosis of RP as assessed by history and photoelectric plethysmography during cooling and rewarming,[18] and have digital pitting scars and/or ulceration. Patients had to fullfill the American College of Rheumatology criteria for SSc.[1] Inclusion criteria otherwise were no prior use of bosentan and the use of a reliable method of contraception during the study. Patients were not eligible in case of digital ulcers due to other conditions than SSc, prostanoid therapy during the month preceding study entry, sympathectomy of the upper limb performed within 12 months before study entry, moderate to severe hepatic impairment (Child Pugh class B or C), baseline values of liver aminotransferases greater than 3 times the upper limit of normal, systolic blood pressure <85 mm Hg, body weight < 40 kg, hemoglobin < 5.5 mmol/l, receiving cyclosporin A, glibenclamide or tacrolimus within 1 week prior to screening or expecting to receive any of these medications during the study, pregnancy or breastfeeding, current smoking or smoking within 4 weeks before study entry, and any

other severe concurrent illness. All patients had to provide written and oral informed consent. Medication used for the treatment of SSc was allowed but had to remain unchanged from 3 months prior to study entry untill the end of the study. Vasodilators, such as calcium channel antagonists and ketanserin, and other oral medications for the treatment of RP, such as angiotensin converting enzyme inhibitors, were allowed and had to be continued at the same dosage throughout the study. In case of progression of digital ischemia, i.e. progression of digital ulceration or critical ischemia, the experimental drug could be stopped and converted to conventional therapy, that is rest and prostanoids infusion. These patients were followed during the study period, but assessments were not continued. All patients except one were recruited during the months of December and January. One patient was enrolled at the beginning of February.

Study design

The study was an observational study. Ethical approval for the study was obtained from the Medical Ethical Committee of the University Medical Center Groningen, the Netherlands. The study started with a 2-4 week pretreatment period, followed by 16 weeks of treatment, and a 4 week posttreatment follow-up. Patients entering the study started with bosentan orally, 62 mg bid for 4 weeks, followed by the target dose of 125 mg bid for 12 weeks. In case liver aminotransferases increased more than 3 times the upper limit of normal the study drug was temporarily discontinued and reintroduced to the initial low dosage regimen after return of the liver enzymes to baseline levels. In case of persistent or recurrent elevation of liver aminotransferases patients dropped out. Noninvasive vascular assessments were done at baseline and at 8, 16 and 20 weeks after start of the study medication. Data from diary were collected prior to start of the study drug and at 2 weekly intervals.

Clinical outcome measurements

Diary

Each day the patient had to make a diary entry for the number, duration and severity of the Raynaud attacks. The overall severity of the Raynaud attacks were measured by means of the Raynaud's Condition Score (RCS), which is a visual analogue scale of 0-10. In this scale '0' means no burden and '10' means very severe burden due to the Raynaud's condition. Completed diaries were collected at each return visit. The mean daily number, the mean daily duration and the mean RCS were calculated for 2 weeks periods during the study.[19] In this study the daily parameters were defined available when data from at least 7 days from a 2 week period were available. A decrease in RCS of 2 points was considered clinically significant. The mean ambient temperature of each day was added to the diary (Dutch national weather report).

Photoelectric plethysmography during cooling and rewarming

Photoelectric plethysmography (PEP) during cooling and rewarming was performed as previously described.[18;20;21] Changes of the blood volume due to pulsations in smaller arteries were recorded in the distal phalanges of all 5 fingers of the right hand. The right hand was placed in a waterbath. The hand was warmed to 33 ºC, and the mean amplitude of the 5 fingers was regarded as the starting value. Subsequently, plethysmograms were

recorded at each step during cooling of the waterbath by steps of 3 ºC every 4 minutes to a temperature of 12 ºC. For the analysis of the cooling phase we used the period of "physiological" changes, that is the sum of the amplitudes at 24 ºC, 21 ºC, 18 ºC, 15 ºC and 12 ºC, expressed as percentages of the starting value of the plethysmography at 33 ºC. This sum was regarded as the area under the curve (AUC) during cooling. During the rewarming phase, plethysmograms were recorded every minute during exposure to room temperature of 25 ºC for 10 minutes. The measured amplitudes during this phase were also expressed as percentages of the starting value of the plethysmography at 33 ºC. The AUC during rewarming was defined as the sum of amplitudes observed during these 10 minutes.

Statistical analysis

Analysis of the changes in clinical outcome measurements from baseline to week 8 and 16 and comparison with week 20 were performed with a paired samples t-test. A two sided p value of <0.05 was considered significant.

RESULTS

A total of 15 patients were enrolled in this study. Baseline characteristics are shown in table 1. The study population consisted of 1 man and 14 women, with a mean age of 52 years (range 34-70). All had limited cutaneous systemic sclerosis, with a mean disease duration of 5.1 years. The mean duration of RP was 11.7 years. The mean modified Rodnan Skin Score[22] was 8.0 and the mean Medsger's severity scale[23] at baseline was 6.5. Ischemic digital ulcers were present at the onset of the study in one patient. In 5 patients there was a history of ischemic digital ulcers. During the last year before study entry 5 patients (33%) were regularly treated with parenteral prostanoids. A total of 2 patients discontinued study medication because of elevations of liver aminotransferases up to 3-fold the upper limit of normal. One patient dropped out at week 4 and one at week 12. From the first drop out patient follow-up data are incomplete. One patient who used methotrexate because of concomitant rheumatoid arthritis, experienced a flare of the arthritis during the study period. Such adverse event has been described before.[24] Other adverse events during the study period are shown in table 2. The effect on digital ulcers will be discussed seperately.

Duration of RP attacks

During the baseline period, the mean daily duration of RP was 62.3 minutes (± 47.3 minutes). Halfway the treatment period, at week 8, the mean daily duration of RP was reduced to 30.0 minutes (± 8.7 minutes, $p=0.003$) and at the end of the study, at week 16, to 11.6 minutes (± 12.1 minutes, $p=0.001$). After discontinuation of bosentan at week 16 this effect was sustained during the 4-week follow-up period. The mean daily duration of RP at week 20 was 13.3 minutes (± 13.7 minutes), not significantly different from week 16 (Figure 1A).

Table 1. Baseline characteristics

Characteristic	N=15
Sex – no. (%)	
Male	1 (7)
Female	14 (93)
Age – yr	
Mean ± SD	52 ± 12.1
Range	34 – 70
Weight – kg	65.7 ± 7.7
Type of systemic sclerosis – no (%)	
Diffuse cutaneous systemic sclerosis	0
Limited cutaneous systemic sclerosis	15 (100)
Antibodies – no (%)	
centromere	8 (53)
nRNP	1 (7)
antinuclear antibodies, not specified	6 (40)
Duration of systemic sclerosis – yr	
Mean ± SD	5.1 ± 2.9
Range	1 - 10
Duration of Raynaud's phenomenon – yr	
Mean ± SD	11.7 ± 7.6
Range	2 - 26
Modified Rodnan Skin Score	
Mean ± SD	8.0 ± 5.1
Range	4 - 22
Medsger's severity scale	
Mean ± SD	6.5 ± 2.7
Range	4 - 14
Previous treatment with parenteral prostanoids before the start of bosentan – no. (%)	5 (33%)
Concomitant treatment – no. (%)	
Calcium-channel blockers	10 (67)
Angiotensin converting enzyme inhibitors	6 (40)
Ketanserin	5 (33)

Number of RP attacks

The mean daily number of RP attacks decreased from 2.9 (± 1.9) at baseline to 1.8 (± 1.5, $p<0.001$) at week 8, and 0.9 (±1.2, $p<0.001$) at week 16. At week 20 no significant increase was seen in daily number of RP attacks (1.2 ± 1.7) (Figure 1B).

Raynaud's condition score

After 8 weeks of treatment the RCS decreased from 4.78 ± 2.55 to 3.55 ± 2.48 ($p=0.002$). At the end of the study period a clinically significant reduction of 2 points was reached. The RCS at week 16 was 2.70 ± 2.13 ($p=0.003$). In the 4 weeks of follow up no significant difference in RCS was noted (Figure 1C).

Table 2. Adverse events

Adverse event	N (%)
hepatotoxicity, >3 ULN*	2 (13.3)
headache	2 (13.3)
panaritium, infected calcinosis	2 (13.3)
amputation because of osteomyelitis and digital ulcus	1 (6.7)
flushing	1 (6.7)
dizziness	1 (6.7)
cholecystolithiasis	1 (6.7)
arthritis flare	1 (6.7)
dyspepsia	1 (6.7)
none	5 (33.3)

*ULN means upper limit of normal

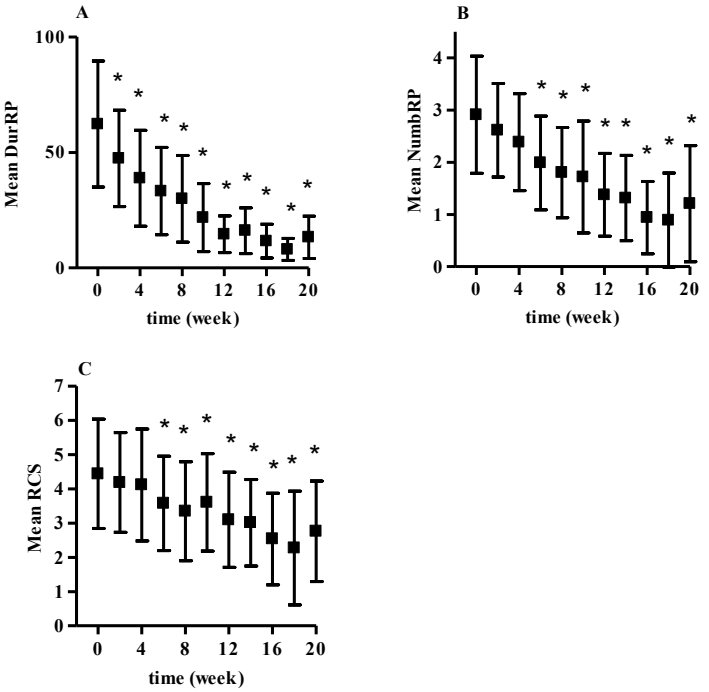

Figure 1. The effects of bosentan on (**A**) mean daily duration (DurRP) in minutes, (**B**) mean daily number (NumbRP), and (**C**) Raynaud's condition score (RCS) of Raynaud's phenomenon attacks. Bars show the 95% confidence interval. * P<0.05 compared to baseline

Daily outdoor temperature

The mean daily outdoor temperature from baseline to week 10 was similar for all patients (2.7 ± 1.1 °C at baseline vs 4,7 ± 3,5 °C at week 8, p=0.073), but increased significantly during the remaining 6 weeks of treatment (12.2 ± 3.5 °C at week 16) and the 4 weeks of follow-up (13.7 ± 3.2 °C at week 20) (Figure 2).

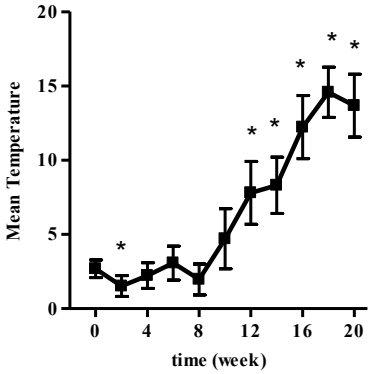

Figure 2. The mean daily outdoor temperature.
Bars show the 95% confidence interval. * P<0.05 compared to baseline.

Photoelectric plethysmography during cooling and rewarming

The plethysmograms showed no significant difference between the area under the curve at week 8 and 16 compared to the area under the curve at baseline for the cooling phase as well as the rewarming phase (Figure 3, Table 3). In other words, no improvement was observed with bosentan treatment during measurements with standardized photoelectric plethysmography. Also, when using the mean temperature at which the plethysmograph showed loss of digital pulsatile flow (amplitude < 4%) no statistical difference was seen between week 16 (loss of flow at 21.7 ± 3.3 °C) and baseline (loss of flow at 20.4 ± 3.3 °C, p=0.560).

Table 3. Photoelectric plethysmography during cold exposure and rewarming. Mean (SD) of the area under the curve (AUC) for each temperature from 24 °C to 12 °C, and for the 10 minutes of rewarming, taking the amplitude at 33 °C as the 100% reference point. Comparisons have been made with baseline.

Cooling	AUC	P value
Baseline	54 (54)	
Week 8	77 (68)	0.339
Week 16	46 (71)	0.776
Week 20	102 (157)	0.252

Rewarming	AUC	P value
Baseline	128 (184)	
Week 8	69 (76)	0.306
Week 16	227 (539)	0.658
Week 20	282 (404)	0.366

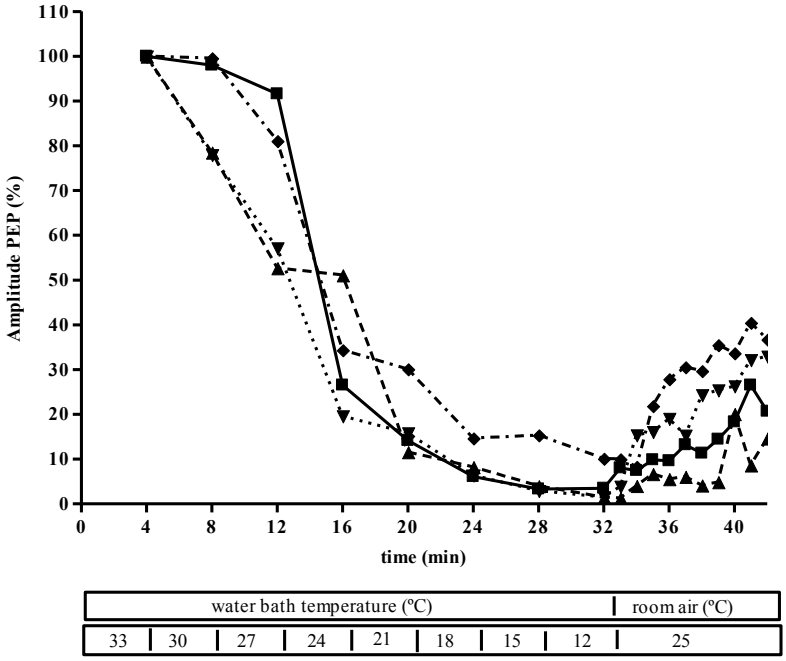

Figure 3. Photoelectric plethysmography (PEP) during cooling and rewarming. Mean values at baseline (■), week 8 (▲), week 16 (▼) and week 20 (♦).

Digital ulcers

At study entry one patient presented with a digital ulcus. Although the mean number and duration of RP attacks and the RCS decreased in this patient, the ulcus did not change. At week 8 amputation proximal to the proximal interphalangeal phalanx of the second finger was necessary because of co-existence of osteomyelitis. One patient developed digital ischemia with necrosis in the presence of infected calcinosis. The finger was surgically explored. Because of persistently elevated liver aminotransferases bosentan had to be stopped a few days before. Conventional therapy with rest and prostanoids infusion was started.

DISCUSSION

This observational study shows an encouraging improvement in frequency, duration and severity of RP attacks in patients with SSc treated with bosentan. This improvement is also reflected in the lack of infusions of prostanoids needed because of severe RP. Previous studies have shown conflicting data regarding the effect of bosentan on RP. The RAPIDS-1 study failed to show an effect on RP, but the primary outcome was prevention of digital ulcers. In this double-blind, placebo-controlled study 122 patients with SSc and a history of

a digital ulcer 12 months prior to study entry were included.[17] Other authors showed in their case-reports or observational studies improvement in RP in patients with SSc. Selenko-Gebauer et al. investigated the effect of bosentan in patients with severe RP associated with SSc and pre-scleroderma. The latter was defined as RP associated with structural alterations of the nailfold capillaries and with disease-specific anti-nuclear antibodies (ANA). Three patients received bosentan during a period of 16 weeks in winter season. These authors found a subjective improvement of RP and an improvement in peripheral thermoregulation by means of thermography after 16 weeks of treatment.[25] Dunne et al. presented a patient with severe RP in SSc who received bosentan because of digital ulceration and gangrene. Besides its beneficial effect on ischemic lesions, pain decreased and peripheral blood flow improved.[26] Ramos-Casals et al. reported four patients with severe RP with or without digital ulceration and SSc in whom RP and the digital ulcers improved during treatment with bosentan.[27] Differences in results in these studies can be explained by the small number of patients included, the design of these observational studies or by differences in the extent of microvascular damage present in the patients.

In most therapeutic trials in RP a diary, recording number, duration and severity of RP attacks, is used. No gold standard for the objective measurement of digital blood flow is available, though several non-invasive vascular techniques are used. Despite significant changes in symptoms of RP previous studies could not all demonstrate an objective improvement in blood flow.[28-36] In this study we used photoelectric plethysmography during cooling and rewarming and, as in other therapeutic trials, we were unable to show a significant change in response to cold provocation and rewarming following bosentan treatment. The study showed intra- and interindividual variability. Reproducibility of photelectric plethysmography has not been studied, but other studies showed limited value of finger skin temperature, laser Doppler fluxmetry and nailfold capillaroscopy during cold provocation tests because of intraindividual variations and moderate reproducibility.[37;38] Also, variability exist between studies in outcome parameters after cold provocation, such as temperature versus blood flow. By measuring flow some techniques only measure capillary blood flow, while others also measure flow in deeper vessels and arteriovenous anastomosis. So, although these techniques may be useful to diagnose RP, they may not be sensitive enough to detect changes as a result of drug therapy.

One might argue that the subjective improvement in RP in our study could be explained by the increase in outdoor temperature. However, during the first 10 weeks of the study period, the cold season, outdoor temperature was stable. Patients already experienced in this period a significant improvement in RP. Otherwise, within 3 months after discontinuation of bosentan at week 16, despite the summer season, parenteral prostanoids were needed in 3 patients because of severe RP or digital ulceration (data not shown). Previous studies have shown that RP is symptomatic throughout the year,[39] and is even more common during transition months (spring, fall);[40] also, other factors, like anxiety, are related to the severity of RP attacks.[41]

Regarding digital ulcers, the RAPIDS-1 study showed an effect of bosentan in preventing new digital ulcers in patients with SSc and an improvement in hand function, but the present study was not designed to show an effect on ulcer healing or preventing new digital ulcers.[17] All patients had digital pitting scars or a history of digital ulcers, though one patient entered the study with a digital ulcus. Bosentan could not prevent amputation

because of underlying osteomyelitis. Also, bosentan treatment could not prevent ulceration in a patient with secondarily infected calcinosis. Other factors, like underlying infection, probably counteracted the vasodilatory effect of bosentan in these patients.
In summary, the results of this observational study show a significant clinical improvement of RP secondary to SSc following bosentan treatment. These results warrant a randomised, controlled study on a larger group of patients with RP secondary to systemic sclerosis.

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CHAPTER 3

NO EFFECTS OF BOSENTAN ON MICROVASCULATURE IN PATIENTS WITH LIMITED CUTANEOUS SYSTEMIC SCLEROSIS

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ABSTRACT

Objective: The endothelium derived vasoconstrictor molecule endothelin-1 (ET-1) has been suggested to play a role in the pathogenesis of Raynaud's phenomenon (RP) and systemic sclerosis (SSc). We studied the effect of bosentan on microvascular structure and function in patients with RP secondary to SSc in a mechanistic pilot study.
Methods: In this single center, open study, 15 patients with limited cutaneous SSc (lcSSc) were treated with bosentan for 16 weeks with a follow-up period of 4 weeks. Changes in microvascular structure and function were studied with assessment of vasodilatory microvascular responses using laser Doppler fluxmetry combined with iontophoresis, capillary permeability using fluorescence videomicroscopy, nailfold capillary microscopy, and serological markers of endothelial activation.
Results: No significant changes were seen in vasodilator responses to acetylcholine and sodium nitroprusside following bosentan treatment. No effect was noted on capillary permeability during treatment. The number of nailfold capillaries remained unchanged. The endothelial activation marker vascular cell adhesion molecule did not change during treatment, but levels of thrombomodulin significantly decreased after 12 weeks of treatment.
Conclusion: Bosentan did not induce significant changes in vasodilator responses, capillary permeability and capillary density during treatment, so no evidence was obtained for structural improvement of microvascular structure and function in this short-time mechanistic pilot study in patients with lcSSc.

INTRODUCTION

Raynaud's phenomenon (RP) is present in more than 95% of patients with systemic sclerosis (SSc) and is often the first manifestation of the disease.[1;2] Endothelial cell dysfunction, associated with microvascular and macrovascular abnormalities, is a hallmark of SSc. Signs of endothelial dysfunction include increased capillary permeability,[3;4] dysregulation of vascular tone[5-7], and elevated markers of endothelial cell injury, such as von Willebrand Factor (vWF), trombomodulin (TM), and soluble vascular adhesion cell molecule-1(s-VCAM-1).[8-13]

Endothelin-1 (ET-1), a potent vasoconstrictor, has been suggested to play a role in the pathogenesis of RP and SSc. Increased levels of ET-1 have been found in patients with primary and secondary RP.[14-19] Recently, treatment with bosentan, an ET-1 receptor antagonist, has been shown to be effective in preventing new digital ulcers in patients with SSc. No improvement was seen in symptoms of RP.[20] We found an encouraging improvement in frequency, duration and severity of RP attacks in 15 patients with SSc who used bosentan during a period of 16 weeks. However, our study was not placebo-controlled and no objective improvement in blood flow was seen.[21]

We hypothesized that the beneficial effects of bosentan in the prevention of digital ulcers could be explained by its effects on microvascular structure and function in this condition. To test this hypothesis we assessed acetylcholine-dependent vasodilatory responses using laser Doppler fluxmetry with iontophoresis, capillary permeability using fluorescence videomicroscopy, nailfold capillaroscopy and markers of endothelial activation, at baseline, during treatment, and after discontinuation of bosentan in patients with SSc and severe RP.

PATIENTS AND METHODS

Patients

Patients from the outpatient clinic with a diagnosis of Raynaud's phenomenon secondary to systemic sclerosis were eligible for the study. Patients had to be at least 18 years old, have a diagnosis of RP as assessed by history and photoelectric plethysmography during cooling and rewarming,[22] and digital pitting scars and/or ulceration. Patients had to fullfill the American College of Rheumatology criteria for systemic sclerosis.[23] Patients were subclassified in subsets as defined by LeRoy et al.[1] Inclusion criteria otherwise included no prior use of bosentan and the use of a reliable method of contraception during the study. Patients were not eligible in case of digital ulcers due to other conditions than SSc, prostanoid therapy during the last month, sympathectomy of the upper limb performed within 12 months before study entry, current smoking or smoking within 4 weeks before study entry, and any other severe concurrent illness. All patients had to provide written informed consent. Medication used for the treatment of SSc was allowed and had to remain unchanged from 3 months prior to study entry until the end of the study. Vasodilators, such as calcium channel antagonists and ketanserin, and other oral medications for the treatment of RP, such as angiotensin converting enzyme inhibitors, were allowed and were continued at the same dosage throughout the study. A total of fifteen patients, all with limited

cutaneous SSc, were recruited. All patients were enrolled during the months of December and January, except for one, who enrolled the study at the beginning of February. No differences in outdoor temperature were seen until week 12. Baseline characteristics of the patients are shown in table 1. To compare the results regarding endothelial markers and the results of laser Doppler fluxmetry and iontophoresis, fifteen historical age- and sex-matched healthy subjects were included as controls.

Table 1. Baseline characteristics

Characteristic	N=15
Sex – no. (%)	
Male	1 (7)
Female	14 (93)
Age – yr	
Median (Range)	52 (34 – 70)
Type of systemic sclerosis – no (%)	
Limited cutaneous	15 (100)
Duration of systemic sclerosis – yr	
Median (Range)	4 (1-10)
Duration of Raynaud's phenomenon – yr	
Median (Range)	10 (2-26)
Immunosuppressive agents – no (%)	
Current use	
Methotrexate	4 (27)
Former use	
Methotrexate	1 (7)
Cyclophosphamide	1 (7)
Azathioprine	1 (7)
Never	8 (53)
Concomitant treatment – no. (%)	
Calcium-channel blockers	10 (67)
Angiotensin converting enzyme inhibitors	6 (40)
Ketanserin	5 (33)
Statins	2 (13)
Aspirin	6 (40)
Coumarin	2 (13)

Study design and drug administration

The study was an open study. Ethical approval for the study was obtained from the Medical Ethical Committee of the University Medical Center Groningen, the Netherlands. There was a 2-4 week pretreatment period, followed by 16 weeks of treatment and a 4 week posttreatment follow-up. Patients entering the study started with bosentan orally, 62.5 mg bid for 4 weeks, followed by the target dose of 125 mg bid for 12 weeks. Clinical outcome measurements were recorded at baseline, week 8, week 16 and week 20, except for the endothelial markers. The laboratory assessments were performed every 4 weeks.

Clinical outcome measurements

Laser Doppler Fluxmetry in combination with iontophoresis

Microvascular endothelium-dependent and endothelium-independent vasodilation was evaluated with laser Doppler fluxmetry (LDF) combined with iontophoresis of acetylcholine (ACh) and sodium nitroprusside (SNP) as described previously.[24] Briefly, after a baseline recording of 5 minutes at rest (baseline flux), iontophoresis was started by setting time and current strength based on previously documented and accepted protocols of multiple fixed doses.[25-27] ACh response was measured at the dorsal side on the middle phalanx of the third finger of the dominant hand and SNP on the same site on the nondominant hand. ACh (1%, Miochol, IOLAB, Bourneville Pharma, The Hague, the Netherlands) was delivered in 7 doses of 0.1 milliamperes (mA) using an anodal current for 20 seconds, with a 60-second interval between each dose to achieve a plateau phase, whereas SNP (0.1%, dissolved in NaCl 0.9%) was delivered in 9 doses of 0.2 mA using a cathodal current for 20 seconds with a 60-second interval between each dose. The measurements were performed sequentially. The order of ACh and SNP delivery was random. The mean flux of the first 5 minutes excluding moving artefacts made up the baseline flux. The plateau flux was defined as the mean flux during the last 2 minutes of the plateau phase. Absolute increase was defined as the difference between plateau flux and baseline flux. Relative increase (vasodilation) was defined as the ratio of absolute increase and baseline flux. Previous studies found a day-to-day variation coefficient for ACh-mediated vasodilation of 10-16%, and for SNP-mediated vasodilation of 8-16%, in healthy subjects on 2 occassions.[26;28;29] We found comparable variation coefficients, i.e. 16% for ACh-mediated vasodilation and 18% for SNP-mediated vasodilation, as determined in 10 individuals on 2 separate days.

Capillary permeability using fluorescein diffusion with 'large field' fluorescence videodensitometry

Large-window sodium fluorescein (NaF) videodensitometry was used to measure skin capillary permeability according to the method described by Jager et al.[30] The system consists of an epiillumination microscope (Olympus BHMJ, Tokyo, Japan) to which a 75 W Xenon lamp (Osram XBO, Berlin, Germany) is mounted. Emitted light is filtered using a fluorescence filter set (Olympus BH2-UDMB, excitation 380-490 nm, barrier 515 nm, Tokyo, Japan). A 2 by 3 mm section of the skin of the medial malleolus of the ankle was visualized (magnification x100). Immersion oil (Leitz, din 58884, Wetzlar, Germany) was applied to the skin to increase transparency. A bolus of NaF solution (0.3 ml of a 15% NaF solution per litre of estimated blood volume) was injected intravenously. The epiillumination microscope visualizes the rapid capillary appearance and the subsequent interstitial leakage of NaF. Images were recorded by a video camera (Grundig FA-85, Fürth/Bay, Germany), in which an automatic gain function was removed, and a S-VHS video recorder. Images were digitized from tape recording (Data Translation 2862 framegrabber with Iris software) after first appearance of the dye every second for 20 minutes. The fluorescence light intensity (FLI) of each image was computed and expressed in arbitrary units. One baseline image was digitized to obtain background FLI, which was subtracted from subsequent intensities. Individual maximum intensity (I_{max}) was set at 100%. All other intensities were expressed as percentages of I_{max}. The average relative

intensity over the first 7 minutes ($I_{av}(7)$) after appearance of the dye was used as a parameter of NaF transcapillary and interstitial diffusion. This value showed a day-to-day variability, expressed as coefficient of variation, of 10%.[30] Dye arrival time (DAT) was defined as the interval from injection of the dye until appearance in skin capillaries.[31]

Nailfold capillary microscopy

An epiillumination microscope (Olympus BHMJ, Tokyo, Japan) was used for nailfold capillary microscopy. The finger of the subject to be examined was placed under the microscope. All studies were performed in a temperature controlled room (24°C). The nailfolds of the middle finger and ringfinger of both hands were examined by widefield microscopy after acclimatization to room temperature for 15 minutes. Immersion oil (Leitz, din 58884, Wetzlar, Germany) was applied to the finger to increase transparency of the skin. Images were recorded by a video camera (Grundig FA-85, Fürth/Bay, Germany) and a S-VHS video recorder. The tapes were evaluated separately by two observers by using a previously described protocol.[32] Numbers of capillary loops in a defined area of 3 mm in the middle of the distal row of nailfold capillaries were counted. The mean score of two observers was given. Patterns of the visible capillary loops were not scored.

Blood analysis

Serum and plasma samples for measuring endothelial markers were stored at -20ºC until analysis. Serum levels of vascular cell adhesion molecule 1 (s-VCAM-1, R&D Systems, Abingdon, UK) and thrombomodulin (TM, Diaclone, Besançon, France) were measured according to the manufacturer's instruction. Von Willebrand factor (vWF) was determined using in-house enzyme linked immunosorbent assays (ELISA) as described before.[33]

Statistical analysis

Values are expressed as mean ± SD. In the analysis of the laser Doppler fluxmetry with iontophoresis the Wilcoxon signed rank test was used. NaF leakage curves were compared by paired t-tests using the area under the curve (AUC) of the first 500 seconds. Analysis of the other NaF leakage parameters, number of nailfold capillaries, and endothelial markers before, during and after treatment was performed with a paired samples t-test. Differences between patients and age- and sex matched healthy controls for LDF with iontophoresis and endothelial markers were assessed by Student's t-test or Mann-Whitney-U as appropriate. A two-sided p-value of <0.05 was considered significant.

RESULTS

Laser Doppler Fluxmetry in combination with iontophoresis

Compared to age-and sex-matched controls a significantly decreased ACh-mediated (endothelial-dependent) vasodilation was found in patients before treatment with bosentan (187 ± 154% (mean ± SD) in patients vs 595 ± 448 % in controls, p= 0.001). SNP-mediated (endothelial-independent) vasodilation was comparable between SSc patients and controls (402 ± 413% vs 637 ± 759%, p= 0.138).

An increased baseline and plateau flux was seen after 16 weeks of treatment. This improvement was not reflected in, or accompanied by a significant increase in ACh-mediated vasodilation nor in SNP-mediated vasodilation (Table 2, Figure 1). No significant difference was found in vasodilation during treatment or after discontinuation of bosentan.

Table 2. Results of laser Doppler flowmetry and iontophoresis

	Before treatment N=15	Week 8 N=14	Week 16 N=12	Week 20 N=9
ACh-mediated vasodilation				
Baseline flux (PU)	32.1 ± 30.5	47.0 ± 37.4	56.4 ± 45.4*	33.3 ± 38.3
Change from pretreatment values		16.9 ± 42.5	24.7 ± 32.5	-2.42 ± 51.7
Plateau flux (PU)	71.0 ± 46.2	96.8 ± 61.8	123.2 ± 93.8*	69.2 ± 60.5
Change from pretreatment values		31.9 ± 76.4	55.8 ± 88.1	7.3 ± 67.7
Absolute increase (PU)	39.0 ± 35.1	49.2 ± 38.8	66.8 ± 65.3	35.9 ± 33.3
Change from pretreatment values		15.0 ± 52.2	31.1 ± 60.3	9.8 ± 44.8
ACh-mediated vasodilation (%)	187.3 ± 154.4	142.2 ± 202.6	116.2 ± 84.2	142.8 ± 124.7
Change from pretreatment values		-47.0 ± 264.3	-81.0 ± 127.2	-31.3 ± 189.9
SNP-mediated vasodilation				
Baseline flux (PU)	23.8 ± 15.8	21.1 ± 14.1	36.8 ± 25.9	51.6 ± 22.9**
Change from pretreatment values		-3.3 ± 22.1	12.1 ± 28.7	22.8 ± 30.6
Plateau flux (PU)	85.2 ± 60.7	56.4 ± 70.0	106.3 ± 102.5	112.1 ± 48.2
Change from pretreatment values		-29.9 ± 84.1	13.4 ± 107.3	29.1 ± 74.4
Absolute increase (PU)	61.4 ± 52.9	35.3 ± 61.7	69.5 ± 86.2	57.5 ± 64.4
Change from pretreatment values		-25.7 ± 69.2	1.3 ± 94.2	-2.6 ± 84.7
SNP-mediated vasodilation (%)	402.0 ± 413.0	155.0 ± 155.4**	197.8 ± 171.5	145.7 ± 130.0
Change from pretreatment values		-244.2 ± 426.4	-235.3 ± 436.7	-151.3 ± 321.2

ACh, acetylcholine; PU, arbitrary units of flux; SNP, sodium nitroprusside

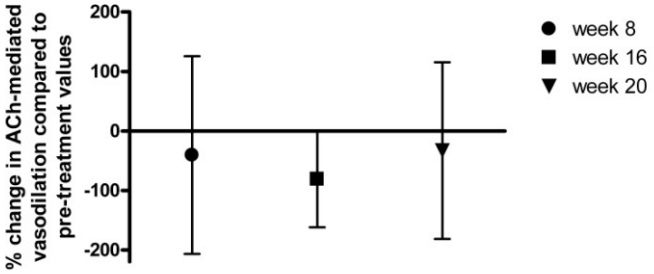
Values are expressed as mean ± standard deviation.

* p=0.05 compared to before treatment, ** p<0.05 compared to before treatment

Fluorescence videomicroscopy

The averaged sodium fluorescein leakage curves, obtained before treatment, and at week 8, week 16, and week 20, are shown in figure 2. The curves for the relative fluorescence light intensity were comparable before, during and after discontinuation of bosentan, although after 8 weeks, when comparing the area under the curve, a trend towards increased NaF leakage (p=0.051) was observed (table 3). At the same time, a shorter, though not significantly, dye arrival time (DAT) was found (p=0.196), suggesting an improvement in skin blood flow. The results of the other capillary sodium fluorescein leakage parameters are also summarized in table 3. The average relative fluorescence light intensity ($I_{av}(7)$) did not significantly change during the study. The DAT showed a trend to be longer after 16 weeks of treatment (p=0.075) and was significantly longer at week 20 (p=0.044) when compared to baseline. No difference was seen in DAT between week 16 and 20. The prolonged DAT at week 20 suggests a reduced skin blood flow, but no differences were seen in $I_{av}(7)$ and the curve for relative fluorescence light intensity as compared to baseline.

a)



b)

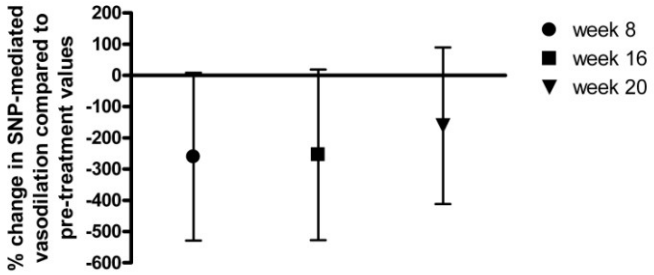


Figure 1. a) Acetylcholine-mediated and **b)** sodium nitroprusside-mediated vasodilation during the study period (week 8 and 16 of bosentan treatment) and 4 weeks after discontinuation of bosentan (week 20) compared to pre-treatment vasodilation. Symbols represent the mean; lines represent the 95% confidence interval.

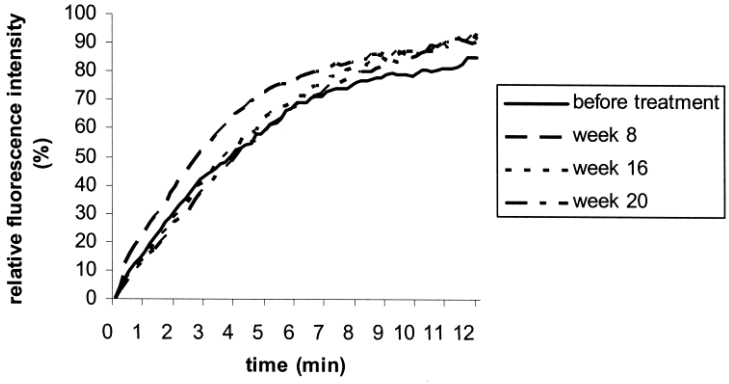


Figure 2 . Relative fluorescence light intensity (%) after NaF arrival in the skin of patients with systemic sclerosis before treatment, at week 8 and 16 of bosentan treatment, and at week 20 (4 weeks after discontinuation of bosentan).

Table 3. Comparison of the NaF leakage curves before treatment, at week 8 and 16 of bosentan treatment, and at week 20 (4 weeks after discontinuation of bosentan).

	Before treatment	Week 8	Week 16	Week20
n	14	14	12	11
AUC	2448 ± 637	2918 ± 611	2522 ± 421	2381 ± 549
Dye arrival time (s)	41 ± 22	32 ± 30	45 ± 23	52 ± 17*
$I_{av}(7)$ (%)	43.9 ± 12.1	46.0 ± 29.0	44.6 ± 8.6	41.4 ±11.0

AUC, area under the curve; $I_{av}(7)$, average relative fluorescence light intensity over the first 7 minutes Values are expressed as mean ± standard deviation.

* $p<0.05$ compared to baseline

Nailfold capillary microscopy

The number of capillaries did not change significantly during the study period. After 16 weeks of treatment 10.2 ± 6.3 capillaries/3mm were seen compared to 9.7 ± 6.4 capillaries/3 mm before treatment with bosentan. Other capillaroscopic features were not noted because of low total number of capillary loops.

Endothelial markers

Concentrations of VCAM-1 and vWF were not increased in patients with SSc compared to age- and sex-matched healthy controls (285 ± 84 vs 252 ± 47 ng/ml, p=0,171, and 66 ± 57 vs 119 ± 191 %, p=0.387 respectively). Concentrations of TM were increased in patients with SSc compared to healthy controls (4.8 ± 4.2 vs 3.4 ± 3.2 ng/ml, p=0.026). VCAM-1 levels did not change during treatment with bosentan. TM levels significantly decreased after 12 weeks of treatment and remained stably decreased at the end of the study period and after discontinuation of bosentan. vWF was significantly increased at week 4 and 20 compared to pre-treatment values, but was not significantly different from pre-treatment values at week 8, 12 and 16 (Table 4).

Table 4. Endothelial markers before treatment, during treatment and after discontinuation of bosentan

	s-VCAM-1 (ng/ml)	TM (ng/ml)	vWF (%)
Before treatment	289 ± 83	4.8 ± 4.2	66 ± 57
Week 4	304 ± 110	4.5 ± 2.7	151 ± 86*
Week 8	292 ± 94	4.3 ± 3.2	101 ± 76
Week 12	291 ± 94	2.1 ± 3.3*	96 ± 103
Week 16	282 ± 76	2.7 ± 2.4*	95 ± 93
Week 20	282 ± 92	3.2 ± 3.7*	147 ± 78*,#

s-VCAM-1, serum vascular cellular adhesion molecule -1; TM, thrombomodulin; vWF, von Willebrand Factor. Values are expressed as mean ± standard deviation.

* $p<0.05$ compared to baseline, # $p<0.05$ compared to week 16

CHAPTER 3

DISCUSSION

This study is unique in its evaluation of the effects of bosentan on several outcome parameters of microvascular structure and function. Bosentan treatment resulted, in these patients with lcSSc and severe RP, as previously has been described in our pilot study, in a significant improvement of RP attacks.[21] However, bosentan did not result in any significant changes in microvascular endothelial-dependent and endothelial-independent vasodilation, capillary permeability, and total number of capillary loops. The endothelial marker s-VCAM-1 also remained unchanged, only a decrease in TM was observed during treatment.

Another important finding from this study was the observation that a reduced microvascular vasodilatory response to ACh was found in our patients, reflecting vasodilatory endothelial dysfunction. The endothelium-independent vasodilatory response to SNP was comparable to that in healthy controls. This supports not only the presumed microvascular endothelial dysfunction in SSc patients, but also the choice of bosentan as an obvious candidate for the treatment of RP in these patients.

During the last decades, laser Doppler fluxmetry (LDF) has been used for evaluating skin microcirculation. LDF provides an estimate of blood flow through the skin.[34;35] Combining LDF with iontophoresis gives the opportunity to investigate the endothelial-dependent (ACh) and endothelial–independent (SNP) vasodilatory flow responses in the digits or forearm.[35] Impaired microvascular function or endothelium dysfunction, demonstrated as decreased endothelium-dependent microvascular reactivity, has been found in patients with hypertension, diabetes mellitus, obesity and preeclampsia.[24;26;28;29;36-38] Reduced endothelium-dependent vasodilatory responses were observed in SSc patients.[39;40] However, some authors did not find differences in vasodilatory flow responses between patients with SSc, patients with primary RP and healthy control subjects.[5;41] This might be explained by methodological differences, like site differences or a different protocol. In our study we used a protocol with a fair day-to-day reproducibility, expressed as coefficient of variation, of about 10-15%.[26;28;29] Another possible explanation for the lack of improvement in endothelial-dependent vasodilation might be a reduced vasodilatory reserve of the skin microcirculation in patients with SSc, as previously hypothesized.[39]

In contrast to our findings, improvement of endothelial function in patients with SSc treated with bosentan was found by Sfikakis et al,[42] as shown by a significant increase in brachial artery ultrasound-derived flow-mediated dilation (FMD) following bosentan treatment. The discrepancy in the treatment effect of bosentan on endothelial dysfunction might be explained by differences in vascular bed, and in technique used. To study endothelial function in conduit arteries, ultrasound has been used to measure FMD of the brachial artery, while LDF is used for measurement of flow in the microcirculation. Comparison between these two different non-invasive methods has revealed conflicting results.[43;44] However, our study does also confirm the results found by Sfikakis et al.,[42] since no positive effect of bosentan on peripheral flow reserve and forearm blood flow using venous occlusion plethysmography was observed in this study, probably due to irreversible structural changes since most patients had a well-established disease. Focal stenosis and occlusion in digital arteries, e.g. the proper digital arteries and ulnar artery, are

common manifestations in SSc patients.[45;46] Our results are consistent with a previous report, in which treatment of bosentan during 24 weeks did not reveal a significant improvement in endothelial-dependent and endothelial-independent vasodilation of the digits.[47]

In patients with SSc, transcapillary leakage of NaF has usually been determined at the nailfold capillaries. A major limitation of this technique in intervention studies has been the large variability in leakage between visualized capillaries. In previous studies we adapted this technique using large-window videodensitometry with sodium fluorescein (NaF) of the forefoot[48] or medial ankle[30;49] to reduce this variability and make it a suitable tool for intervention studies. Using this technique, our study showed no improvement during bosentan treatment in total number of capillaries and in transcapillary leakage. A trend to increased NaF leakage was seen after 8 weeks of treatment together with a non-significant decrease in dye arrival time (DAT), suggesting bosentan-related increase in total skin blood flow. However, no differences in NaF leakage parameters were present after 16 weeks of treatment. Such relatively minor changes in permeability, and the increased DAT at week 20, suggest that these changes are caused by a decrease in blood flow, since DAT is mainly determined by skin blood flow. A difference in temperature cannot be held responsible since room temperature and local skin temperature remained stable.

In our study, endothelial activation markers did not show consistent changes during treatment with bosentan. We did find a significant decrease in TM from week 12 till the end of the study. This result suggests improvement of endothelial dysfunction, i.e. decrease in endothelial cell damage, especially since baseline values were increased compared to healthy controls. Otherwise, we did not find a difference in levels of s-VCAM and vWF between SSc patients and healthy controls. Soluble adhesion molecules, like s-VCAM-1 are increased particularly during the early edematous phase of the disease.[11] The fact that none of our patients had early or active disease might explain lack of differences, but is also consistent with a previous report in which bosentan did not result in changes of ET-1, soluble intercellular adhesion molecule 1 (ICAM-1) and E-selectin.[42] The observed increase of vWF at week 4 and 20 could be related to cold exposition shortly before[50;51] as some patients had been exposed to cold more than 30 minutes prior to blood collection, though skin temperature had returned to normal at this time point. Levels of s-VCAM and TM were not supposed to be influenced by cold exposition.[51;52]

Obviously, the major limitation of our study is its uncontrolled and open design and its relatively short duration. Also, patients were allowed to continue other vasodilating agents besides prostanoids. Therefore, a possible effect of bosentan on vasodilating responses and capillary permeability might be reduced. On the other hand, we found no positive effect of bosentan on microvascular structure and function. Since placebo is not supposed to be an effective therapy, the chance to find a beneficial effect of bosentan in a placebo-controlled trial in a short-time study is not to be expected. One might argue that seasonal variation could have influenced the results of the vascular studies. Although we cannot completely rule out a seasonal effect, outdoor temperature remained stable in all patients until week 12. Another limitation is the drop out percentage of 20-40% at the end of the study period. In most cases this was due to logistic reasons.

In conclusion, the findings of this observational study indicate that use of the endothelin-1 receptor antagonist does not induce changes in vasodilator responses, capillary

permeability and capillary density during a period of 16 weeks treatment in patients with lcSSc. As we found a decrease in TM, some limited improvement of endothelial cell dysfunction cannot be ruled out. A prospective controlled study, possibly with a longer follow-up is warranted in order to examine the efficacy of bosentan on functional and structural abnormalities in the microvasculature in patients with early SSc.

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CHAPTER 4

IMPAIRED MICROVASCULAR REACTIVITY IN PATIENTS WITH SYSTEMIC SCLEROSIS IS DUE TO THE DISEASE ITSELF

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ABSTRACT

Objective: To assess endothelial function at the level of skin microvasculature in patients with systemic sclerosis (SSc). Furthermore, to evaluate whether microvascular reactivity is related to the disease or traditional cardiovascular risk factors.

Methods: Microvascular skin reactivity was assessed using a combination of laser Doppler fluxmetry (LDF) and iontophoretic administration of acetylcholine (ACh, endothelium-dependent vasodilator) and sodium nitroprusside (SNP, endothelium-independent vasodilator) in 47 SSc patients and 23 healthy controls. Traditional risk factors for cardiovascular disease and serum markers for endothelial activation were determined.

Results: Median (interquartile ranges) ACh-mediated vasodilation, expressed as a percentage increase in flux, was significantly decreased in SSc patients compared to controls (168% (68-288) versus 596% (303-899), $p<0.001$), as was SNP-mediated vasodilation (165% (69-343) versus 443% (250-831), $p=0.001$). Impaired Ach-mediated vasodilation was found also after adjustment for confounders in a linear regression analysis ($p=0.003$), but impaired SNP-mediated vasodilation in SSc patients was not statistically significant anymore after adjustment for confounders ($p=0.076$).

Conclusion: This study demonstrates impaired endothelium-dependent vasodilation in SSc patients, also after adjustment for cardiovascular risk factors. Besides endothelial dysfunction, structural defects may also present in the microcirculation of SSc patients since we demonstrated a tendency to impaired endothelium-independent vasodilation.

INTRODUCTION

Systemic sclerosis (SSc) is a systemic autoimmune disease characterized by immunologic abnormalities, fibrosis of the skin and internal organs, and widespread vascular involvement. Vascular or endothelial cell dysfunction has been suggested to be a crucial element in the pathogenesis of SSc,[1-3] suggested by, amongst others, the presence of Raynaud's phenomenon (RP) in allmost all patients, digital ulcers and nailfold capillaroscopic abnormalities. Several non-invasive techniques are available for the assessment of endothelial cell dysfunction. Laser Doppler fluxmetry combined with iontophoresis of vasoactive agents provides a noninvasive method to assess endothelium-dependent and endothelium-independent vasodilator responses of the microcirculation.[4;5] Skin fibrosis was found not to interfere with iontophoretical diffusion of vasoactive drugs across the skin in SSc patients compared with healthy controls, although, as expected, impaired vasodilator responses were observed. [6] Until now, different protocols studying endothelial cell dysfunction in SSc patients have been used with conflicting results. In general, these studies showed impaired endothelium-dependent and endothelium-independent vasodilation in patients with SSc, the endothelium-dependent vasodilation being most affected.[6-11] Endothelial cell dysfunction has also been found in patients with classical traditional risk factors like hypertension, diabetes mellitus and obesity.[4;5;12-16] Therefore, the aim of our study was to assess endothelial microvascular function in patients with SSc in comparison to healthy control subjects, and to relate the results to possible confounding variables, such as the presence of hypertension and obesity, and to disease related factors.

MATERIALS AND METHODS

Patients

From November 2005 to May 2006, 47 consecutive SSc patients from our out-patient clinic were included. Patients with SSc were classified according to the ACR criteria[17] and were subclassified in subsets as defined by LeRoy et al.[18;19] Disease activity was assessed by the preliminary European Scleroderma Study Group (EScSG) activity indices (a score ranging from 0-10). A score higher than 3 denotes active disease.[20;21] Also, the revised preliminary SSc severity scale (Medsger's severity scale), a measure of activity, damage and severity, was used. This scale is a 9-organ disease severity scale in which for each organ system a score of 0 to 4 is applied, with 0 being normal and 4 denoting endstage organ involvement.[22] Pregnancy and diabetes mellitus were exclusion criteria. Healthy subjects in the same age decade were included as controls. Ethical approval for the study was obtained from the Medical Ethical Committee of the University Medical Center Groningen. Informed consent was obtained from each participant.

Data were obtained from all subjects with respect to traditional risk factors for cardiovascular disease (CVD), including body mass index (BMI), smoking status, blood pressure, lipid levels and family history of CVD (considered positive if first-degree relatives suffered from CVD before the age of 60 years). Sitting blood pressure was measured by a mercury sphygmomanometer (Korotkov V) at the end of the examination.

Laser Doppler fluxmetry in combination with iontophoresis

Skin perfusion was measured by a Periflux 4000 laser Doppler system in combination with a Periflux tissue heater (PF4005, Peritemp; all equipment from Perimed, Stockholm, Sweden) set to 31 ºC. The measurements were performed between 8:00 am and 5:00 pm in a quiet room with a temperature of approximately 24 ºC. Caffeine-containing drinks or smoking were not allowed from 12 hours before the test. A special iontophoresis probe (PF481-2, Perimed, Stockholm, Sweden) containing a thermostatic probe holder was placed on the dorsal side of the middle phalanx of the third finger. A battery-powered iontophoresis controller (Perilont 382, Perimed, Stockholm, Sweden) was used to provide a direct current for drug iontophoresis. Iontophoresis is a non-invasive method of introducing charged substances across the surface of the skin by means of a small direct electric current. Iontophoresis of acetylcholine (ACh, 1%, Miochol, IOLAB, Bourneville Pharma, The Hague, the Netherlands) induces vascular smooth muscle relaxation indirectly via nitric oxide (NO) release and is considered an endothelium-dependent vasodilator. Iontophoresis of sodium nitroprusside (SNP, 0,1%, dissolved in NaCl 0,9%), a NO donor, evokes vasodilation by directly increasing cyclic guanosine monophosphate (cGMP) in vascular smooth muscle cells, and is considered an endothelium-independent vasodilator. We followed the same protocol as decribed previously.[23;24] ACh was delivered with an anodal current; seven doses (0.1 mA for 20 s) were delivered, with a 60-second interval between each dose. SNP was delivered using a cathodal current; nine doses (0,2 mA for 20 s) were delivered, with a 90-second interval between each dose. The mean flux of the first 5 minutes excluding moving artefacts made up the baseline flux. The plateau flux was defined as the mean flux during the last 2 minutes of the plateau phase. Absolute increase was defined as the difference between plateau flux and baseline flux. Relative increase (vasodilation) was defined as the ratio of absolute increase and baseline flux. This protocol gave intraindividual coefficients of variation for maximal responses of 16% for ACh and 18% for SNP, as determined in 10 individuals on 2 separate days.[24]

Blood analysis

Lipid levels were measured by routine techniques. Total cholesterol, triglycerides and HDL-cholesterol were determined by enzymatic methods on a Vitros 950 (Ortho-Clinical Diagnostics, Rochester NY, USA). HDL-cholesterol was isolated by precipitation of LDL-cholesterol and VLDL with phosphotungstate and magnesiumchloride. Serum LDL-cholesterol was calculated using the Friedewald formula, excluding patients with triglycerides levels above 5.0 mmol/l.

Additional serum and plasma samples for determination of markers of endothelial activation were stored at -20ºC until analysis. Serum levels of vascular cell adhesion molecule-1 (s-VCAM-1, R&D Systems, Abingdon, UK) and thrombomodulin (TM, Diaclone, Besançon, France) were measured according to the manufacturer's instruction. Von Willebrand factor (vWF) was determinded using in-house enzyme linked immunosorbent assays (ELISA) as described.[25]

Statistical analysis

Because of a low percentage of diffuse cutaneous SSc (dSSc) in our patient group, the 2 subsets were taken together in the analysis. Data are expressed as mean ± SD or median

(interquartile range) unless stated otherwise. Differences between patients and controls were assessed by Student's t-test, by Mann-Whitney-U, and by X^2 as appropriate. The correlation between endothelial-dependent and endothelial-independent vasodilation and disease related variables was assessed by Pearson correlation coefficient or by Spearman correlation coefficient as appropriate. Multiple regression analysis was used to assess the relationship between vasodilator responses, the presence of SSc, and demographic and clinical characteristics. An unadjusted analysis in which no corrections were made for confounders, and an adjusted analysis in which corrections were made for confounders are presented. A variable was defined to be a confounder when significantly changing the regression coefficient B in the model testing the assocoiation between patient category (SSc patients and healthy controls) and vasodilator response (ACh-medited vasodilation and SNP-mediated vasodilation). Due to lack of normal distribution of residuals in the model, ACh-mediated and SNP-mediated vasodilation values had to be transformed by using the natural logarithm of these values +1. The variables age, gender and BMI were also studied as a potential effect modifier in the relationship of interest. A 2-tailed p-value of <0.05 was considered significant. All analyses were carried out with the Statistical Package of Social Science, version 12.1. for Windows (SPSS, Inc, Chicago, IL)

RESULTS

Characteristics of patients and controls

Clinical characteristics of the subjects are presented in table 1. A total of 47 patients (7 males, 40 females) with SSc participated in the study, with a mean age of 55 years (± 12 years). Limited cutaneous SSc (lcSSc) was present in 43 (91%) and diffuse cutaneous SSc (dcSSc) in 4 (9%) patients. Patients with SSc had a median disease duration of 7 years (IQR 3-12) and had experienced Raynaud's phenomenon (RP) for almost 12 years (IQR 6-25). The median Rodnan Skin Score was 6.0 (IQR 4.0-13.0), the preliminary European Scleroderma Study Group activity index (EScSG) was 0.5 (IQR 0.5-1.5) and the Medsger's severity Scale was 6.0 (IQR 5.0-7.0). The 23 healthy controls were younger (mean age 48 ± 10 years, p=0.012) than SSc patients. Patients and controls were similar with respect to current smoking habits (6% vs 0%, p=n.s.), but were different regarding former smoking habits. No differences were seen in family history of CVD, but patients had a higher prevalence of treated hypertension. No differences were present in systolic and diastolic blood pressures between patients and controls. However, antihypertensive medication or vasodilating drugs for Raynaud's phenomenon were significantly more frequently used in SSc patients in comparison with controls. Patients had also lower HDL-cholesterol and higher triglycerides levels than healthy controls. No differences were found in levels of endothelial activation markers.

Table 1. Clinical characteristics of patients with systemic sclerosis and healthy controls

	Controls N=23	SSc N=47
Gender, male sex (n (%))	3 (13)	7 (15)
Age (years)	48 ± 10	55 ± 12
Ever smoking (n (%))	1 (4)	23 (49)*
Family history CVD (n (%))	4 (17)	10 (21)
Treated hypertension	0 (0)	11 (23)
SBP (mm Hg)	120 (110-125)	120 (110-135)
DBP (mm Hg)	78 (72-81)	75 (70-80)
Antihypertensive or vasodilating medication (n (%))	0 (0)	38 (81)*
Calcium channel blockers (n (%))	0 (0)	27 (57)*
Angiotensin converting enzyme inhibitors (n (%))	0 (0)	12 (26)*
Prostanoids < 4 weeks before study	0 (0)	2 (4)
BMI (kg/m ^{2})	23.3 ± 2.5	23.7 ± 2.9
Total cholesterol (mmol/l)	5.16 ± 0.88	5.29 ± 0.96
HDL-cholesterol (mmol/l)	1.84 (1.58-2.07)	1.40 (1.23-1.83)*
LDL-cholesterol (mmol/l)	2.74 ± 0.92	3.09 ± 0.82
Triglycerides (mmol/l)	0.94 (0.71-1.31)	1.36 (1.17-2.14)*
Statins (n (%))	0 (0)	5 (11)
VCAM-1 (ng/ml)	275 ± 59	254 ± 89
vWF (%)	58 (46-101)	73 (31-125)
TM (ng/ml)	2.8 (2.1-3.7)	3.8 (2.4-5.0)

SSc, systemic sclerosis; CVD, cardiovascular disease; DM, diabetes mellitus; BMI, body mass index; SBP, systolic blood pressure; DBP, diastolic blood pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; VCAM-1: vascular cell adhesion molecule-1; vWF: von Willebrand factor; TM: thrombomodulin

Unless stated otherwise, values are expressed as mean ± standard deviation when normally distributed and as median (interquartile range) when non-normally distributed; *:$p<0.05$

Vascular responses

Responses to ACh and SNP are shown in table 2 and figures 1-2. Baseline flux did not differ significantly between SSc patients and controls. In SSc patients a significantly reduced ACh-mediated ($p<0.001$) and SNP-mediated vasodilation ($p=0.001$) were present compared to controls.

Table 2. Results of microvascular measurements

	Controls N=23	SSc N=47
Acetylcholine		
baseline flux (PU)	25 (13-35)	24 (14-41)
plateau flux (PU)	155 (96-219)	61 (31-113)*
absolute increase (PU)	113 (82-176)	33 (13-64)*
ACh-mediated vasodilation (%)	596 (303-899)	168 (68 -282)*
Sodium nitroprusside		
baseline flux (PU)	16 (10-33)	23 (10-35)
plateau flux (PU)	112 (80-151)	67 (34-85)*
absolute increase (PU)	92 (54-117)	29 (15-65)*
SNP-mediated vasodilation (%)	443 (250-831)	165 (69-343)*

SSc, systemic sclerosis; PU, arbitrary units of flux; Ach, acetylcholine; SNP, sodium nitroprusside
Values are expressed as median (interquartile range); * :p<0.01

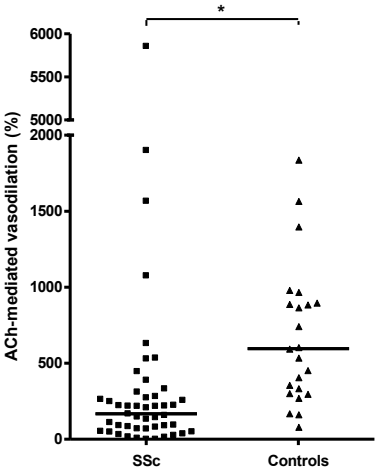

Figure 1. Acetylcholine-mediated vasodilation in patients with systemic sclerosis (▲) and controls (•). Bars represent median values. * p<0.01 versus controls.

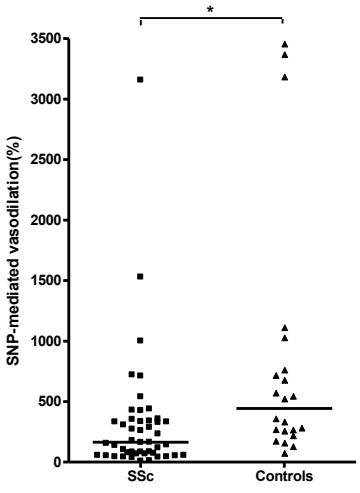

Figure 2. Sodium nitroprusside-mediated vasodilation in patients with systemic sclerosis (▲) and controls (•). Bars represent median values. * p<0.01 versus controls.

Since baseline characteristics between SSc patients and controls were not similar (table 1), we conducted a linear regression analysis with (the transformed value of) ACh-mediated vasodilation and SNP-mediated vasodilation as dependent variables (Table 3). The adjusted model, corrected for ever smoking, use of antihypertensive medication and age as confounders, revealed that the difference in ACh-mediated vasodilation between SSc patients and healthy controls was explained just by the presence of SSc (p=0.003). The association between SSc and SNP-mediated vasodilation was not statistically significant (p=0.076) after correction for HDL cholesterol, use of antihypertensive medication and age as confounders. For both ACh-mediated vasodilation and SNP-mediated vasodilation no correction for the other parameters (BMI, gender, LDL cholesterol, total cholesterol and triglycerides) was necessary, since these outcome parameters were no confounders in the model. No effect modification by age, gender or BMI was found either.

In patients an inverse correlation (ρ= -0.338, p=0.027) was found for ACh-mediated vasodilation and modified Rodnan Skin Score, suggesting a more impaired vasodilation with increasing stiffness of the skin. No such correlation was found for other disease related factors in ACh-mediated vasodilation or SNP-mediated vasodilation (Table 4). Also, no correlation was found between ACh-mediated vasodilation or SNP-mediated vasodilation and endothelial activation markers (data not shown).

Table 3 Linear regression analysis for **a)** endothelium-dependent (ACh-mediated) vasodilation and **b)** endothelium-independent (SNP-mediated) vasodilation in SSc patients and healthy controls

a)

Group (patients, controls)	B[#]	95% confidence interval	p-value
Unadjusted/crude	1.393	0.677; 2.109	<0.001
Adjusted*	1.632	0.494; 2.771	0.006

The natural logarithm of ACh-mediated vasodilation +1 was used as dependent variable as using ACh-mediated vasodilation showed non-normal distribution of residuals in the linear regression model.

* In the adjusted models corrections were made for the confounders age, ever smoking and use of antihypertensive; a total of 68 patients and controls were available for this analysis

[#] B is the regression coefficient

b)

Group (patients, controls)	B[#]	95% confidence interval	p-value
Unadjusted/crude	1.087	0.510; 1.665	<0.001
Adjusted*	0.851	-0.090; 1.792	0.076

The natural logarithm of SNP-mediated vasodilation +1 was used as dependent variable as using NP-mediated vasodilation showed non-normal distribution of residuals in the linear regression model.

In the adjusted models corrections were made for the confounders age, ever smoking and use of antihypertensive; a total of 60 patients and controls were available for this analysis

[#] B is the regression coefficient

Table 4. Correlations between acetylcholine- and sodium nitroprusside-mediated vasodilation and disease related variables

	ACh		SNP	
	ρ	p-value	ρ	p-value
Disease duration	0.158	0.289	-0.086	0.568
Duration Raynaud's phenomenon	0.008	0.956	-0.011	0.941
Modified Rodnan Skin Score	-0.338	0.027	-0.152	0.331
Medsger's severity score	-0.265	0.086	-0.131	0.403
EScSG activity index	-0.245	0.097	-0.190	0.201
Cumulative prednisolone dose	0.074	0.764	-0.128	0.601

SSc, systemic sclerosis; ACh, acetylcholine; SNP, sodium nitroprusside; EScSG: European Scleroderma Study Group

DISCUSSION

The present study demonstrates, as expected, impaired endothelium-dependent responses in patients with SSc compared to healthy controls. These decreased endothelium-dependent responses were present even after adjustment of confounders, known for their influence on microvascular reactivity. Endothelium-independent responses in SSc patients were impaired, yet not statistically significant after adjustment of confounders.

Endothelial dysfunction is thought to be crucial in the pathogenesis of SSc. Previous studies, using different techniques and protocols, showed conflicting results, but, in general, impaired endothelial-dependent and endothelial-independent vasodilatation was observed.[6-11;26-28] However, few of these studies mentioned baseline characteristics besides age and gender, and when mentioned, results of microvascular reactivity were not corrected for possible confounders.

Our results are in agreement with previous studies in SSc using the same [6;11] or a different non-invasive method.[26-29] After adjustment for possible confounders we still found a difference in endothelial-dependent vasodilation between patients and healthy controls. In contrast to our results, Anderson et al. did not find differences in vascular reactivity in patients with SSc and primary Raynaud's phenomenon measured in forearm skin[7] and digital skin,[8] using laser Doppler fluxmetry in combination with iontophoresis. As stated before, these differences can be explained by the non-invasive method used, but also by differences in dermal site or iontophoresis protocol. We used the same concentration of ACh but a lower concentration of SNP compared to the studies mentioned above. Also differences were present in iontophoretic currents, frequency and interval between each dose. In our protocol iontophoresis was started by setting time and current strength based on previously documented and accepted protocols of multiple fixed dosages.[4;5;16;30]

Our results are in agreement with the observation that endothelial abnormalities are found in patients with early SSc. [31] We expected to find endothelial abnormalities since our patients had a median disease duration of 6 years. The impaired vasodilatory response to ACh reflects endothelial dysfunction. For endothelial-independent vasodilation no functioning endothelium is required, since SNP is a donor of NO, normally produced by the intact endothelium. Although no statistical significance was reached in the linear regression

analysis, looking at the p-value found and the relatively low number of participants, we cannot rule out that there is a structural or functional abnormality of the smooth muscle of the arteriolar wall.

An unavoidable limitation in our conclusion is that antihypertensive and vasodilating medication was used in almost all SSc patients and could have influenced vasodilating properties, both endothelium-dependent and endothelium-independent. We corrected the data for use of antihypertensive and vasodilating medication in the linear regression analysis. Using digital arteriography focal stenosis and occlusions of digital arteries have been found in almost all patients with SSc.[32-35] Finally, a decreased capillary density, as seen in the nailfolds of SSc patients, might have contributed to impaired vasodilatory responses.[36] Assessment of capillary density by nailfold capillaroscopy together with assessment of skin microcirculation evaluated by iontophoresis of ACh and SNP in combination with laser Doppler fluxmetry in SSc patients has not been investigated yet.

The negative correlation between ACh-mediated vasodilation and Modified Rodnan skin score is interesting. The iontophoresis probe was placed on the dorsal side of the middle phalanx of the third finger, when possible on normal skin. None of the patients had an oedematous phase of the disease, but a fibrotic or atrophic skin was present in part of our patients with mostly the limited cutaneous subset. Therefore, the question remains whether decreased ACh-mediated vasodilation is explained by a decreased diffusion of ACh due to sclerodactyly, or by a true fault in the microcirculation of the digits. However, the observation that only the ACh-mediated vasodilation and not the SNP-mediated vasodilation correlated negatively with the Rodnan skin score, argues against decreased diffusion of the drugs as an explanation for the impaired vasodilation, as also has been demonstrated by La Civita et al.[6]

In conclusion, this study confirms that ACh-mediated (endothelium-dependent) vasodilation in patients with SSc is impaired, also after adjustment for cardiovascular risk factors, such as age, smoking, blood pressure and dyslipidaemia. A tendency to impaired SNP-mediated (endothelium-independent) vasodilation was found after correction for confounders. This suggests that, besides endothelial dysfunction, also structural defects or functional changes in the arteriolar wall might be present which are due to the disease itself. This subject should be further investigated, since early therapeutic intervention in systemic sclerosis and Raynaud's phenomenon might influence the development of structural defects or arteriolar changes in the arteriolar wall.

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CHAPTER 5

DECREASED CAPILLARY PERMEABILITY AND CAPILLARY DENSITY IN PATIENTS WITH SYSTEMIC SCLEROSIS USING LARGE-WINDOW SODIUM FLUORESCEIN VIDEODENSITOMETRY OF THE ANKLE

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ABSTRACT

Objective: Local **c**apillary permeability in patients with systemic sclerosis (SSc) has been reported increased when assessed by nailfold capillaroscopy. We measured capillary permeability at a clinically less affected site by using large-window fluorescein videodensitometry of the ankle. We hypothesized that increased capillary permeability or leakage is a generalized phenomenon in SSc.

Methods: Large-window videodensitometry with sodium fluorescein was performed in 38 SSc patients and 20 healthy controls. Capillary permeability was expressed as the average relative light intensity over the first 7 minutes ($I_{av}(7)$) after appearance of fluorescein in skin capillaries.

Results: Capillary permeability, expressed as $I_{av}(7)$ was significantly decreased in patients with SSc (47.3 ± 15.0% vs 57.6 ± 9.4% in controls, p=0.007), as was capillary density (12 ± 6 /mm^2 vs 26 ± 11/mm^2, p<0.001). Adjustment for capillary density in multivariate regression analysis demonstrated that differences in $I_{av}(7)$ between SSc patients and controls were related to differences in capillary density, BMI and HDL cholesterol.

Conclusion: At the level of the ankle decreased capillary permeability was found in SSc patients, related to decreased capillary density. Microvascular involvement in SSc is widespread, but no evidence was established for increased capillary permeability at the level of individual capillaries as a generalized phenomenon.

INTRODUCTION

Systemic sclerosis (SSc) is a systemic autoimmune disease characterized by immunologic abnormalities, fibrosis of the skin and internal organs, and widespread microvascular involvement. Clinical features of vascular involvement include Raynaud's phenomenon (RP), but also major complications like pulmonary hypertension and renal crisis. Vascular or endothelial dysfunction has been suggested to be a crucial element in the pathogenesis of SSc, and increased capillary permeability and vascular leakage is one of its manifestations.[1-3]

Microvascular abnormalities can be detected by nailfold capillaroscopy. These abnormalities in nailfold capillaries in SSc consist of structural alterations of the capillaries and a reduced capillary density. Bushy patterns, extravasates and giant capillaries are present.[4-8] Bollinger et al. found increased capillary permeability in SSc using dynamic fluorescence videomicroscopy of the nailfold.[9] By means of this approach fluorescein leakage was studied at the single capillary level. It is unknown whether increased capillary permeability in SSc is a local or a generalized phenomenon. Sodium fluorescein (NaF) videodensitometry of the ankle, using a 'large-window' technique, visualizes a larger number of capillaries. Using this technique, we found increased NaF leakage as a marker of increased capillary permeability in patients with diabetes mellitus, and especially in those with microalbuminuria.[10-12] The main advantage of this technique is its reproducibility with a mean of the coefficients of variation over 3 experiments of 10% in diabetic patients and controls.[10] This allows the technique to be feasible and useful for intervention studies.

In this study, we investigated microvascular permeability in SSc patients compared to healthy controls by using large-window videodensitometry with sodium fluorescein at the level of the ankle, in order to test the hypothesis that capillary leakage is a generalized phenomenon in SSc.

METHODS

Subjects

Consecutive patients with SSc according to the ACR criteria[13] attending the outpatient clinic of our hospital were asked to participate in this study. All patients suffered from RP, confirmed by a previously observed abnormal response to cooling at plethysmography, and all had anatomical capillary abnormalities at nailfold capillary microscopy. A control group of age matched non-smoking healthy subjects was used who had no RP or any other concomitant disease. Exclusion criteria were pregnancy or lactation, smoking, diabetes mellitus, cardiovascular disease, cerebrovascular disease, peripheral vascular disease, renal failure, hepatic failure and autonomic neuropathy. Data were obtained from all subjects with respect to body mass index (BMI) and lipid levels. Ethical approval was obtained from the Medical Ethical Committee of the University Medical Center Groningen, and all participants provided written informed consent.

Sodium fluorescence videodensitometry

Large-window sodium fluorescein (NaF) videodensitometry was used to measure skin capillary permeability according to the method described by Jager et al.[10] Subjects were studied while lying on their right side. The room temperature was approximately 24 ºC. The local skin temperature of the medial ankle was monitored using a thermocouple (Ellab DU3-s, Copenhagen, Denmark) and kept between 28 ºC and 32 ºC. The system consists of an epiillumination microscope (Olympus BHMJ, Tokyo, Japan) to which a 75 W Xenon lamp (Osram XBO, Berlin, Germany) is mounted. Emitted light is filtered using a fluorescence filter set (Olympus BH2-UDMB, excitation 380-490 nm, barrier 515 nm, Tokyo, Japan). A 2 by 3 mm (6 mm^2) section of the skin of the medial malleolus of the ankle was visualized (magnification x100). Immersion oil (Leitz, din 58884, Wetzlar, Germany) was applied to the skin to increase transparency. A bolus of NaF solution (0.3 ml of a 15% NaF solution per litre of estimated blood volume) was injected intravenously. The epiillumination microscope visualizes the rapid capillary appearance and the subsequent interstitial leakage of NaF. Images were recorded by a video camera (Grundig FA-85, Fürth/Bay, Germany), in which an automatic gain function was removed, and a S-VHS video recorder. Images were digitized from tape recording (Data Translation 2862 framegrabber with Iris software) every second for 20 minutes after first appearance of the dye. Dye arrival time (DAT) was defined as the interval from injection of the dye until appearance in skin capillaries.[12] The number of capillaries visualized in the 2 by 3 mm section was counted 60 sec after appearance of the dye from tape recordings. The fluorescence light intensity (FLI) of each image was computed and expressed in arbitrary units. One baseline image was digitized to obtain background FLI, which was subtracted from subsequent intensities. Individual maximum intensity (I_{max}) was set at 100%. All other intensities were expressed as percentages of I_{max}. The average relative intensity over the first 7 minutes ($I_{av}(7)$) after appearance of the dye was used as a parameter of NaF transcapillary and interstitial diffusion. This value showed a day-to-day reproducibility, expressed as coefficients of variation, of 10%.[10]

Statistical analysis

Data are expressed as mean ± standard deviation, when variables were normally distributed. In case of a non-normal distribution data are reported as median and interquartile ranges. Between-group comparisons were performed using parametric or non-parametric tests. Entire relative NaF leakage curves were compared by ANOVA for repeated measurements. Univariate analysis was performed using Spearman's correlation coefficient. Multiple regression analysis was used to assess the relationship between $I_{av}(7)$, the presence of SSc, and demographic and clinical characteristics. An unadjusted analysis in which no corrections were made for confounders, and an adjusted analysis in which corrections were made for confounders are presented. A variable was defined to be a confounder when significantly changing the regression coefficient B in the model testing the assocoiation between patient category (SSc patients and healthy controls) and capillary permeability ($I_{av}(7)$).Two-tailed p-values <0.05 were considered significant.

RESULTS

SSc patients had a median disease duration of 7 years (IQR 3-12). 90% of patients had limited cutaneous SSc, and 10% diffuse cutaneous SSc. Both subsets were taken together in the analysis because of the low number (4 out of 38) of patients with diffuse cutaneous SSc. Almost half of the patients (47%) had anticentromere autoantibodies, 5% Scl-70 antibodies, 8% no antibodies, and 39% had non-specified antinuclear antibodies. Immunosuppressive agents, such as methotrexate, azathioprine and cyclophosphamide, were used in 32 % of SSc patients during the study, while 16% of patients used corticosteroids. Baseline characteristics of SSc patients and healthy controls are shown in table 1. Significant differences between patients and controls were found in gender, BMI, systolic and diastolic blood pressure, HDL- and LDL-cholesterol. Differences in blood pressure can be explained by the use of vasodilators, such as calcium channel blockers and ketanserin, and other oral medications for the treatment of RP, such as angiotensin converting enzyme (ACE) inhibitors, in SSc patients. Since RP and hypertension were exclusion criteria for healthy controls, use of vasodilating agents was statistically different between groups. No difference was present regarding the use of statins.

Table 1. Characteristics of SSc patients and healthy controls

	SSc patients	Controls	p-value
N	38	20	
Male (n (%))	3 (8%)	7 (35%)	0.023
Age (years)	54.3 ± 11.0	53.2 ± 15.3	0.750
BMI (kg/m^2)	23.7 ± 2.9	27.6 ± 4.4	<0.001
Systolic blood pressure (mm Hg)	120 (110-131)	137 (124-156)	0.004
Diastolic blood pressure (mm Hg)	75 (70-80)	84 (79-93)	0.001
ACE-inhibition therapy (n (%))	6 (16)	0	0.084
Calcium channel blockers (n (%))	22 (58)	0	<0.001
Total cholesterol (mmol/l)	5.39 ± 0.97	6.19 ± 1.81	0.096
HDL-cholesterol (mmol/l)	1.45 (1.23-1.85)	1.07 (0.95-1.37)	0.001
LDL-cholesterol (mmol/l)	3.18 ± 0.79	4.71 ± 1.33	<0.001
Triglycerides (mmol/l)	1.34 (1.05-2.03)	1.15 (0.79-1.87)	0.286
Statins (n (%))	3 (8%)	0	0.544
Skin temperature ankle (ºC)	30.2 ± 1.1	30.1 ± 0.7	0.846
Capillary density (n/mm^2)	12 ± 6	26 ± 11	<0.001
Background intensity (AU)	344 (321-851)	716 (511-1102)	0.005
Dye arrival time (s)	46 ± 26	48 ± 14	0.707
NaF leakage $I_{av}(7)$ (%)	47.3 ± 15.0	57.6 ± 9.4	0.007

Data are expressed as mean ± standard deviation, or as median (interquartile range).

SSc, systemic sclerosis; ACE, angiotensin converting enzyme; HDL, high density lipoprotein; LDL, low density lipoprotein; $I_{av}(7)$, average relative fluorescence light intensity over the first 7 minutes

Capillary density was significantly decreased in patients compared to controls (12 ± 6/mm^2 vs 26 ± 11/mm^2, p<0.001). Also background fluorescence was decreased in SSc patients. Dye arrival time (DAT) was not different between groups. Transcapillary leakage of NaF, expressed as the average relative intensity over the first 7 minutes after arrival of the dye ($I_{av}(7)$), was significantly lower in SSc patients (47.3 ± 15.0 % vs 57.6 ± 9.4 % in controls, p=0.007), supporting reduced capillary permeability (Table 1). The relative fluorescence intensity curve was also significantly lower in SSc patients than in healthy controls (p=0.006, figure 1). Univariate analysis performed using data of all subjects showed only a significant positive correlation between $I_{av}(7)$ and capillary density and BMI. Multivariate analysis disclosed that differences in $I_{av}(7)$ between SSc patients and healthy controls were related to differences in capillary density, BMI and HDL cholesterol (table 2). Addition of other confounders, that is diastolic tension, triglycerides, use of calcium channel antagonists or angiotensin converting enzyme inhibitors, and gender, did not change the outcome.

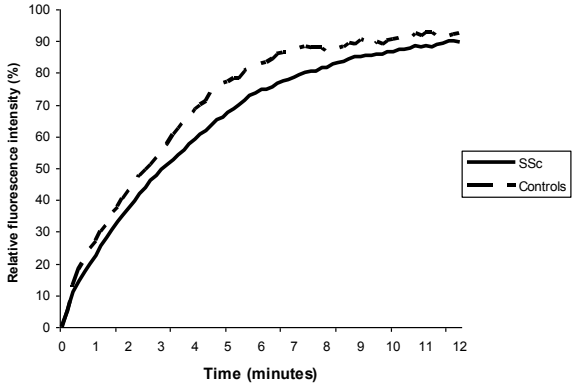

Figure 1. Relative fluorescence light intensity (%) after NaF arrival in the skin of SSc patients (–) and healthy controls (- - -).

Table 2. Linear regression analysis of determinants of NaF leakage ($I_{av}(7)$) between SSc patients and healthy controls

Group (patients, controls)	B#	95% confidence interval for B	p-value
Unadjusted/crude	10.326	2.943; 17.708	0.007
Adjusted*	-12.451	-25.921;1.018	0.069

*In the adjusted model corrections were made for the confounders capillary density/mm^2, BMI and HDL cholesterol; # B is the regression coefficient

DISCUSSION

In the present study, we evaluated capillary permeability in SSc patients at a clinically less affected site by using large-window fluorescein videodensitometry of the ankle in order to test the hypothesis that capillary leakage is a generalized phenomenon in SSc. Unexpectedly, we found a significantly impaired NaF leakage in SSc patients, but this impaired NaF leakage could be explained by a decreased capillary density at the level of the ankle in SSc patients. Compared to SSc patients, higher levels of NaF leakage in healthy controls could be explained by higher BMI and lower HDL cholesterol levels, indicating a more unfavourable cardiovascular risc profile.

In patients with SSc, microangiopathy with increased capillary permeability of the nailfolds is characterized by loss of capillaries, morphologic changes in the capillaries, asymmetric leakage of the dye, partial or complete loss of dye concentration at the halo (i.e, outer border of the skin papilla), and preferential accumulation sites of dye in the remote interstitial space.[9] Most prominent abnormalities in patients with SSc are present in the nailfolds. Probably, the frequent episodes of digital ischemia in SSc patients with often severe Raynaud's phenomenon may be responsible for the reported increase in local nailfold capillary permeability. We found an impaired capillary permeability at the level of the ankle, but also a decreased capillary density. The decrease in capillary density might suggest that microvascular involvement is not restricted to the nailfolds in SSc patients. Limited data is available about capillary density elsewhere in the body in SSc patients. Using labial capillary microscopy, Grassi et al.[14] were not able to find decreased capillary density in SSc patients compared to controls, even in patients with definite avascular areas in the nailfold, whereas patients did show widespread disorganisation of the capillaries. However, evaluating muscle biopsies from SSc patients, Scarpelli et al demonstrated a decreased capillary density compared to patients with polymyositis, rheumatoid arthritis, muscle dystrophy and neurogenic atrophy.[15] In the present study, dye arrival time was similar in patients and controls, suggesting no differences in total skin flow. Since NaF leakage was reduced as was capillary density, we cannot exclude that permeability at a single capillary level was increased, possibly in order to compensate for the decreased capillary density. However, we cannot confirm our hypothesis that increased capillary permeability is a generalized phenomenon.

Since Raynaud's phenomenon is present in more than 95% of SSc patients and microvascular abnormalities, such as a decrease in capillary density, are present in SSc, increased capillary permeability was hypothesized by Grassi et al. to be a protective mechanism at least in the digits. Increased permeability has been supposed to allow enhanced nutritional exchange in the remaining capillary loops, but this hypothesis has not been substantiated further.[16] Cold exposure has been shown to decrease capillary flow, resulting in a significant increase in dye arrival time and pericapillary and interstitial fluorescent light intensities.[17] These cold-induced changes in fluorescent light intensities were counteracted by the use of nifedipine in SSc patients.[18] In the present study, use of vasodilating agents, such as calcium channel blockers and ACE inhibitors, might have influenced NaF leakage, since these agents were significantly more frequently used in SSc patients. ACE-inhibitors are known to reduce NaF leakage, but their use, and also the use of calcium channel blockers, were not correlated with NaF leakage in this study (data not shown).[12]

Although controls with cardiovascular disease and other known risk factors possibly influencing capillary permeability were excluded, significant differences between patients and controls were present in, amongst others, BMI and lipid levels. BMI and HDL cholesterol were confounders in the association between capillary permeability and patient category (SSc patients or healthy controls). Higher levels of capillary permeability in controls compared to SSc patients could also be explained by a more unfavourable cardiovascular risk profile, i.e. higher BMI values and lower HDL cholesterol levels. Low HDL-cholesterol concentration[19;20] and increased weight[21;22] are known to be related with endothelial dysfunction.

The decreased background intensity in patients with SSc was unexpected. In patients with diabetes increased background intensity has been observed, probably related to accumulation of advanced glycosylation end-products (AGEs).[12] Skin autofluorescence, related to the accumulation of AGEs, is strongly associated with progression of coronary heart disease and mortality in patients with diabetes, or those in hemodialysis.[23] Using ELISA, accumulation of Nε-(carboxymethyl)lysine, one of the AGEs, was found increased in SSc, and increased levels were associated with early changes at nailfold capillaroscopy, suggesting involvement of AGEs in SSc.[24] Therefore, we expected to find increased instead of decreased background intensity. AGE-associated fluorescence rises with age and can also be increased in nondiabetic smokers and in various manifestations of cardiovascular disease.[25;26] Because AGE-accumulation and skin autofluorescence are strongly related to collagen-linked autofluorescence, and thereby to the half-life of collagen, the previously reported increases in both collagen synthesis and, especially, increased degradation of skin collagen in SSc suggest that reduced skin autofluorescence in our patients may be related to accelerated skin collagen turnover.[27-29]

In conclusion, our study confirms the presence of more generalized microvascular involvement in SSc as capillary density at the level of the ankle was decreased. The decreased capillary permeability in SSc patients could be explained by the decreased capillary density. Moreover, a more unfavourable cardiovascular risk profile, i.e. increased BMI and lower HDL cholesterol levels, were found in controls. This might also explain higher levels of capillary permeability in controls compared to SSc patients. Using large-window videodensitometry, we can, however, not exclude that increased capillary permeability is present at the level of a single capillary to meet the demands of nutritional exchanges in response to reduced capillary density at the level of the ankle. Therefore, we cannot confirm our hypothesis that increased capillary permeability is a generalized phenomenon in SSc.

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CHAPTER 6

MACROVASCULAR DISEASE AND ATHEROSCLEROSIS IN SYSTEMIC SCLEROSIS

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ABSTRACT

Atherosclerosis is considered a chronic inflammatory disorder. Several autoimmune rheumatic diseases are characterized by premature and accelerated atherosclerosis in which both classical and non-classical risk factors contribute to atherogenesis. Systemic sclerosis (SSc) is characterized by vasculopathy, and microvascular involvement is common. Macrovascular involvement is considered rare, although increased prevalence of macrovascular disease has been reported as well. Here, we review the literature regarding coronary artery disease, cerebrovascular disease and peripheral arterial disease in systemic sclerosis. An increased prevalence of distal peripheral artery disease in the digits has been found. The prevalence of coronary artery disease and cerebrovascular disease is not increased, although studies using intima-media thickness of the carotid artery as marker of early atherosclerosis showed discrepant results. Besides traditional risk factors, as present in the general population, non-traditional risk factors are present in SSc as well, such as increased lipoprotein[a], oxidized LDL, inflammation, vasospasm and endothelial dysfunction. Moreover, markers of vascular damage in atherosclerosis, like antibodies to oxidised LDL, and increased levels of soluble vascular adhesion molecules, have been described in association with vascular damage in SSc. Nevertheless, generalized premature atherosclerosis has not been detected in SSc. Therefore, further research is necessary to assess the prevalence of clinically manifest or subclinical early atherosclerosis in SSc.

INTRODUCTION

Atherosclerosis underlying cardiovascular mortality is the leading cause of death in developed countries (WHO Statistical Information System, www.who.int/whosis). Atherosclerosis is a disease of large and medium-sized arteries and can result in ischemia and infarction. Clinical manifestations include coronary heart disease, stroke and peripheral vascular disease. Atherosclerosis is considered an inflammatory disease, in which, amongst others, monocytes, macrophages, and T-cells as well as autoantibodies, autoantigens, and cytokines play a role.[1;2] Several autoimmune diseases, including rheumatoid arthritis (RA),[3-7] systemic lupus erythematosus (SLE),[8-13] and Wegener's granulomatosis (WG),[14] are characterized by an increased prevalence of atherosclerosis, and, consequently, increased cardiovascular morbidity and mortality.

Systemic sclerosis (SSc) is an autoimmune disorder characterized by widespread vascular involvement. Microvascular abnormalities and Raynaud's phenomenon (RP) are well-known as major sites of pathology,[15-18] but less attention has been paid to macrovascular abnormalities. Although survival in SSc has improved during the last decades, a recently performed meta-analysis still showed increased standardized mortality ratios. Involvement of major organs, as heart, lungs and kidneys, was found to be an independent adverse predictor of mortality.[19]

In this review, we discuss data on the prevalence and etiology of macrovascular disease and atherosclerosis in SSc. Evaluation of the prevalence of clinically manifest atherosclerosis in SSc is difficult, as both the prevalence of SSc in the general population and the number of observed cardiovascular events are low. However, early atherosclerosis can be studied in cohorts of patients using standardized techniques. Therefore, we will also review studies describing subclinical, early atherosclerositic changes as assessed by measuring intima media thickness (IMT) of the carotid artery.[20-25] We searched for original articles in PubMed and Medline published between 1950 and 2007 with the search strategy "atherosclerosis OR macrovascular" in association with "systemic sclerosis". We also used relevant papers retrieved from references of articles found by the search strategy.

Evidence for macrovascular disease and atherosclerosis in systemic sclerosis

The prevalence of vascular abnormalities in SSc has been considered to be inversely proportional to the size of blood vessels studied.[26] Macrovascular disease was considered extremely rare. Although the heart is one of the major organs involved in SSc,[27] coronary arteries were rarely involved in histopathologic material[28-31] or at coronary angiography.[32] Even so, in SSc patients admitted to a hospital because of acute myocardial infarction, the odds ratio of having normal coronary arteries was 33.89 compared to the patients admitted from the general population, suggesting microvascular and not macrovascular disease in these patients.[33] Also, cerebrovascular involvement in SSc has rarely been documented, although the opposite has been stated.[34] Only one retrospective cohort study is available, showing no increased prevalence of cerebrovascular disease in 31 female SSc patients compared with matched controls (prevalence 26% vs 19%, RR 1.3 with 95% confidence interval of 0.5-3.3).[35] More studies are available regarding peripheral atherosclerotic vascular disease. An increased prevalence of peripheral vascular disease in SSc patients compared to healthy controls (21.7% vs 4.6%) has been observed by Veale et al, using a

questionnaire for intermittent claudication,[36] and by Youssef et al, using data available from angiography, Doppler ultrasound or physical examination (prevalence 58% vs 10%, RR 6.0 with 95% confidence interval of 2.0-18).[35] When angiographic findings of the lower and upper limb in SSc patients were related to cardiovascular risk factors an association was observed between these risk factors and proximal peripheral artery disease, but not distal peripheral artery disease.[37] Distal peripheral artery disease is present in the digits of many SSc patients, showing a high frequency of digital stenosis and occlusions in the digital arteries of patients. Lesions were most frequently found in the 2nd to 5th proper palmar digital artery, the ulnar artery and the superficial palmar arch.[38-41] As a consequence, digital ischaemia, ulceration or amputation is a well-known, but feared manifestation in SSc.[42-44]

Several groups have studied subclinical, early atherosclerosis in SSc. Using intima-media thickness (IMT) of the carotid artery as a marker of early atherosclerosis discrepant results were reported. No differences in IMT or intraluminal diameter of the common carotid artery (CCA) between SSc patients and controls were noted by some authors,[45-48] while others found significantly increased IMT values or increased prevalence of carotid artery disease in SSc patients (Table 1).[49-53] IMT values of the femoral artery in SSc patients and healthy controls were comparable.[25;45] Nevertheless, large vessel involvement has been suggested in SSc since altered elastic properties of the carotid artery, increased stiffness of the aorta, and decreased arterial distensibility have all been reported in SSc patients.[45;54-59] Another method to assess subclinical atherosclerosis is by means of ankle brachial pressure index (ABPI), which is also the usual non-invasive assessment approach of patients with symptomatic peripheral vascular disease. This technique can also be used to predict cardiovascular disease and mortality.[60-62] ABPI has been used in asymptomatic SSc patients. Ho et al reported a significantly increased prevalence of peripheral artery disease,[50] but in other studies no differences in ABPI between SSc patients and healthy controls were found.[49;51;60] Several other non-invasive tests have been used for the assessment of subclinical early atherosclerosis. Evaluating of heart rate variability has been used for assessing cardiovascular autonomic function, and is found to be a predictor of sudden arrhythmic death, but also of nonarrhythmic cardiac events.[63] The latter can be explained by the influence of heart rate variability on hemodynamic factors, leading to changes in the vascular wall. Evaluating heart rate variability showed a reduced variability, indicating the presence of autonomic cardiac neuropathy in SSc patients without known cardiac disease.[64-67]

In conclusion, an increased prevalence of distal peripheral artery disease is present in SSc. Conflicting results regarding early signs of atherosclerosis are present, but can be explained by methodological differences, such as differences in patients included in the study, comorbidity, and non-invasive techniques used.

Etiology

Traditional risk factors were equally distributed between SSc patients and controls in the above mentioned studies. Hence, other factors beyond traditional cardiovascular risk factors may contribute to any putatively increased prevalence of cardiovascular, cerebrovascular and peripheral vascular disease in SSc.

Table 1 Carotid ultrasound studies in SSc

First author	No. of SSc patients	lcSSc: dcSSc	Mean age SSc patients, years	No. of controls	Mean age controls, years	IMT values of the CCA (and bulb) in SSc patients compared to controls
Lekakis[52]	12	0:12	49	12	49	0.83 ± 0.3 mm vs 0.46 ± 0.2 mm, p=0.002
Cheng[46]	53	N.A.	43	53	55	0.65 ± 0.24 mm vs 0.63 ± 0.20 mm, p=0.74
Cheng[45]	52	33:19	lcSSc 56 dcSSc 55	52	50	0.68 ± 0.27 mm (lcSSc) and 0.62 ± 0.20 mm (dcSSc) vs 0.63 ± 0.20 mm, p=0.090
Szucs[47]	29	19:10	52	29	49	0.67 ± 0.26 mm vs 0.57 ± 0.09 mm, p=0.067
Kaloudi [51]	66	55:11	lcSSc 62 dcSSc 53	66	58	0.90 ± 0.036 mm (lcSSc) and 0.87 ± 0.043 mm (dcSSc) vs 0.69 ± 0.013 mm, p<0.01
Bartoli[49]	53	45:8	60	53	56	0.85 ± 0.03 mm vs 0.68 ± 0.01 mm, p<0.03
Bartoli[53]	35	24:11	61	20	"matched"	0.93 ± 0.29 mm vs 0.77 ± 0.13 mm, p< 0.003

Abbreviations: No., number; lcSSc, limited cutaneous systemic sclerosis; dcSSc, diffuse cutaneous systemic sclerosis; IMT, intima-media thickness; CCA, common carotid artery; N.A., not available

Lipoprotein profile

Dyslipidemia, i.e. increased levels of low density lipoprotein (LDL) cholesterol and triglycerides, and decreased high density lipoprotein (HDL) cholesterol,[68] is an important traditional risk factor for cardiovascular disease. Decreased HDL levels have been detected in patients with limited cutaneous SSc (lcSSc) compared to healthy controls.[69] No studies are available on LDL levels in SSc. However, patients with SSc showed increased susceptibility to oxidation of LDL, a process in which oxidized LDL (OxLDL) is formed.[70] OxLDL is a proatherogenic lipoprotein, which, amongst others, promotes foam cell formation, vascular oxygen radical formation, tissue remodeling, endothelial dysfunction, and even vasospasm.[71;72] Another cardiovascular pathogenic factor is lipoprotein[a].[73] Its exact mechanism is unknown, but lipoprotein[a] (Lp[a]) counterbalances the pro- and anticoagulant, pro- and anti-inflammatory, and vasorelaxing and vasoconstricting properties of the endothelium, in which raised concentrations are linked with atherosclerosis and thrombosis.[74;75] In patients with SSc, increased concentrations of Lp[a] without further differences in lipid profile in comparison with healthy controls have been found, both in limited cutaneous SSc (lcSSc) and diffuse cutaneous SSc (dcSSc).[76;77]

Autoantibodies

Increased concentration of antibodies to OxLDL (aOxLDL) have been described in atherosclerosis.[78-82] In young individuals or in early stages of atherosclerosis low aOxLDL levels have been found.[83;84] Immunization with OxLDL in experimental animals resulted in a protective effect on the process of atherogenesis leading to increased aOxLDL levels and a reduction in atherosclerosis.[85-89] Increased concentrations of aOxLDL have been found in patients with SSc, particularly in dcSSc.[77;90]

Antiphospholipid antibodies are present in the antiphospholipid syndrome (APS). APS is clinically characterized by recurrent arterial and venous thrombosis as well as pregnancy losses. These antibodies have procoagulant activity and are proatherogenic, as has been shown by increased prevalence of cardiovascular disease in patients with APS and SLE.[91] The role of antiphospholipid antibodies in vascular manifestatations in SSc is unclear. A prevalence of anticardiolipin antibodies (aCL) and anti-beta2-glycoprotein-I antibodies (anti-beta2GPI) of 0-40% has been reported in SSc, but no relation of the antibodies with clinical manifestations of the antiphospholipid syndrome was seen.[92-99] However, some studies found an association with pulmonary hypertension, endothelial dysfunction and myocardial ischemia or necrosis.[92;95;96] No association with digital ischemia was found.[97]

Other antibodies present in diseases with vascular damage are the antiendothelial cell antibodies (aECA). These antibodies have been reported in various diseases, such as coronary atherosclerosis, diabetes mellitus, hypertension, and autoimmune diseases.[100-106] In Wegener's granulomatosis and SLE a relation between aECA and disease activity has been found. However, the precise role of aECAs is unclear. It is possible that aECAs are an epiphenomenon of vascular damage, or are pathogenic antibodies.[107] AECAs have been detected in 22-85% of SSc patients.[108-110] Differences in detection of AECAs may be ascribed to patient selection and technique used in the laboratory. AECAs are also associated with vascular damage in SSc, such as digital ischemia, pulmonary hypertension and nailfold capillary abnormalities.[111-114]

Inflammation

Inflammation is a hallmark of systemic autoimmune diseases. Also in SSc inflammation is present when the disease is active. Disease activity in SSc can be assessed by using the preliminary European Scleroderma Study Group (EScSG) activity indices (a score ranging from 0-10). A score higher than 3 denotes active disease.[115;116] One of the items of this scale is C-reactive protein (CRP), found to be a strong marker of cardiovascular risk in asymptomatic subjects and subjects with a history of a cardiovascular event.[117-122] Elevated acute phase reactants have been found especially in dcSSc (41.8%) compared to lcSSc (24.6%), as have been described in the large EULAR Scleroderma Trials and Research (EUSTAR) group database.[123] Therefore, inflammation might play a role in vascular abnormalities in SSc. No data are available concerning acute phase reactants and atherosclerosis in SSc.

Endothelial dysfunction

Inflammation, auto-antibodies, oxLDL, and other stimuli allow the endothelium to undergo changes resulting in endothelial cell activation. Endothelial cell activation is an initiating step in atherogenesis.[124] Endothelial function can be assessed non-invasively by means of several methods, based on the inability of dysfunctional endothelium to cause vasodilation of the vessels by its inability to release endothelium-derived vasodilatory mediators. Acetylcholine is used in most studies studying endothelium-dependent vasodilation, as acetylcholine cannot exerts its effects in the absence of functional endothelial cells.[125] Endothelium-independent vasodilation can be studied by using glyceryl nitrate, causing vasodilation by direct action on the smooth muscle.[126;127] Measurement of flow mediated dilation (FMD, endothelium-dependent) and endothelium-independent vasodilation via high-ultrasound techniques allowed the detection of endothelial dysfunction in children and adults with risk factors for atherosclerosis.[126] A relationship between endothelial dysfunction, intima media thickness and cardiovascular risk factors has been established.[128] Studies using laser Doppler flowmetry,[129-133] studies using brachial reactivity via ultrasound,[47;52;53;58;134] and studies using venous occlusion plethysmography[135;136] show evidence both in favor of, but also against impaired endothelial-dependent as well as endothelial-independent vasodilation in SSc. This might be explained by methodological differences between studies, like differences in sites of measurements or use of a different protocol. Also, endothelial function of conduit arteries has been studied by the assessment of ultrasound-derived FMD of the brachial artery, while LDF has been used for measurement of flow in the microcirculation. Comparison between these two different non-invasive methods has revealed conflicting results.[137;138] Interestingly, an association between impairments of FMD and increased IMT was found in some[52;53] but not all studies.[47] Expression of vascular adhesion molecules, such as P-selectin, L-selectin, vascular cell adhesion molecule (VCAM)-1 and intercellular adhesion molecule (ICAM)-1, is increased in atherosclerosis, but their role in the recruitment, regulation of transmigration and retention of immune cells to atherosclerotic lesions still needs to be clarified.[139] Increased levels of soluble forms of various adhesion molecules in combination with endothelial dysfunction has also been described in SSc, but not in relation to atherosclerosis or macrovascular disease.[58;140-143]

Vasospasm

Autopsy studies of the heart showed focal areas of acute and subacute necrosis in combination with contraction band necrosis in the presence of normal coronary arteries in SSc, even in patients who, prior to death, were known with signs of ischemic heart disease.[28-31] Bulkley et al stated that contraction band necrosis was the result of a reperfusion lesion, suggesting that the lesions found at autopsy were the consequence of transient nonperfusion due to arrythmias or Raynaud's phenomenon (RP) of the coronary arteries.[28;29] Cold provocation in patients with SSc resulted in reversible myocardial perfusion abnormalities in a significant proportion of patients.[144-146] Indeed, severe impairment of coronary blood flow, not due to coronary artery stenosis, was found in 7 asymptomatic SSc patients using a myocardial multidetector computed tomography.[147] Coronary flow reserve (CFR), a marker of the coronary circulation, is used to evaluate the effects of coronary artery stenosis on coronary microvasculature and myocardial perfusion.

Impaired CFR has been found in SSc patients, but no differentiation could be made between vasospasm or structural abnormalities as underlying factors.[148-150] Improvement of myocardial perfusion after administration of oral nifidepine supports the hypothesis of myocardial RP in SSc.[151-153] Coronary vasospasm is also known in the general population as variant angina or Prinzmetal angina. Endothelial dysfunction as well as local hyperreactivity play a role in its pathogenesis.[154] Vasospasm with or without the presence of structural vascular abnormalities was also seen during digital arteriography in patients with SSc, in whom RP is a common manifestation.[40] Vasospasm has also been observed in patients with SLE, with and without RP, and SSc in cerebral blood flow after a cooling test of the hand.[155]

SUMMARY

The immune system is involved in the pathogenesis of atherosclerosis, which is considered to be an inflammatory disease. Atherosclerosis is more prevalent and the risk of coronary vascular disease is increased in patients with various autoimmune diseases compared to healthy controls. Systemic sclerosis also is an autoimmune disease, and vascular involvement is frequent. Raynaud's phenomenon is often the first manifestation. The disease is mainly characterized by microvascular involvement, and increasing evidence suggests also macrovascular involvement. Besides traditional risk factors, as in the general population, non-traditional risk factors are present in SSc, such as increased Lp[a], OxLDL, inflammation, vasospasm and endothelial dysfunction. Moreover, markers of vascular damage, like aOxLDL, aECA and increased levels of vascular adhesion molecules are present in SSc. However, clinically manifest atherosclerosis was found to be rare, and studies assessing subclinical, early atherosclerosis showed conflicting results. Cardiovascular involvement is most likely the result of vasospasm of the coronary arteries. Further research is necessary to assess the prevalence of clinically manifest or subclinically early atherosclerosis in SSc, and to explain differences in accelerated atherosclerosis between SSc and other autoimmune rheumatic diseases.

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CHAPTER 7

EARLY ATHEROSCLEROSIS IN SYSTEMIC SCLEROSIS AND ITS RELATION TO DISEASE OR TRADITIONAL RISK FACTORS

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ABSTRACT

Objective: Several systemic autoimmune diseases are associated with an increased prevalence of atherosclerosis which could not be explained by traditional risk factors alone. In systemic sclerosis (SSc) microvascular abnormalities are well recognized. Previous studies have suggested an increased prevalence of macrovascular disease as well. We compared patients with SSc to healthy controls for signs of early atherosclerosis by measuring intima-media thickness (IMT) of the common carotid artery in relation to traditional risk factors and markers of endothelial activation.
Methods: 49 patients with SSc, in which 92% had limited cutaneous SSc, and 32 healthy controls were studied. Common carotid IMT was measured by using B-mode ultrasound. Traditional risk factors for cardiovascular disease were assessed and serum markers for endothelial activation measured.
Results: In patients with SSc the mean IMT (median, interquartile ranges; 0.69 mm (0.62-0.79)) was not significantly increased compared to healthy controls (0.68 mm (0.56-0.75, p=0.067). Also, after correction for the confounders age, HDL-cholesterol and LDL-cholesterol (p=0.328), or using a different model taking into account the confounders age, HDL-cholesterol and history of macrovascular disease (p=0.474), no difference in IMT was present between SSc patients and healthy controls. Plaques were found in 3 patients and not in healthy controls (p=0.274). In patients no correlations were found between maximum IMT, disease related variables and markers of endothelial activation. Endothelial activation markers were not increased in SSc patients compared to controls.
Conclusions: SSc is not associated with an increased prevalence of early signs of atherosclerosis.

INTRODUCTION

Systemic sclerosis (SSc) is a generalized connective tissue disorder, characterized by fibrosis of the skin and internal organs and widespread vascular lesions. The pathogenesis of the vasculopathy is not fully understood, but a viral trigger, immune reactions to viral or environmental factors, reperfusion injury or anti-endothelial antibodies may all be involved.[1] Also, angiogenesis is insufficient or defective.[2;3] Most attention has been given to microvascular disease in SSc, but previous studies have suggested an increased prevalence of macrovascular disease as well.[4;5]

In other autoimmune diseases, such as systemic lupus erythematosus (SLE), rheumatoid arthritis and Wegener's granulomatosis, a significantly increased prevalence of atherosclerosis has been described.[6-10] Atherosclerosis is nowadays considered an inflammatory disease in which endothelial cell dysfunction is strongly implicated in its pathogenesis,[11] in part related to traditional risk factors like smoking and dyslipidemia. Because of the increased cardiovascular morbidity and mortality in the aforementioned auto-immune diseases, attention has been given to the presence and treatment of cardiovascular risk factors. Despite increased mortality rates in SSc, partly due to cardiac involvement, cardiovascular risk factors and the presence of macrovascular disease have less been emphasized.[12;13]

In this study, we assessed signs of early atherosclerosis by measuring intima-media thickness of the common carotid artery in patients with SSc and healthy controls. In addition, we related the outcome to traditional risk factors and markers of endothelial activation.

PATIENTS AND METHODS

Patients

Consecutive patients with SSc, according to the ACR criteria,[14] attending our outpatient clinic were included. Patients were subclassified in subsets as defined by LeRoy et al.[15;16] Forty-nine patients were included. Pregnancy was an exclusion criterium. Healthy subjects were included in this study as controls. Ethical approval for the study was obtained from the Medical Ethical Committee of the University Medical Center Groningen. Informed consent was obtained from each participant.

Data were obtained from all subjects with respect to traditional risk factors for cardiovascular disease (CVD), including body mass index (BMI), smoking status, diabetes, blood pressure, lipid levels and family history of CVD (considered positive if first-degree relatives suffered from CVD before 60 years of age).

In patients with SSc we assessed disease related factors as possible determinants of macrovascular disease. To assess disease activity the preliminary European Scleroderma Study Group (EscSG) activity indices (a score ranging from 0-10) was used. A score higher than 3 denotes active disease.[17;18] Also, the revised preliminary SSc severity scale (Medsger's severity scale), a measure of activity, damage and severity, was used. This scale is a 9-organ disease severity scale in which for each organ system a score of 0 to 4 is applied, with 0 being normal and 4 denoting endstage organ involvement.[19] We also

recorded statin use, cumulative prednisolone dose and current or former use of immunosuppressive agents.

Measurements of intima-media thickness

B-mode ultrasonography was used to measure the common carotid intima-media thickness (IMT). Measurements were limited to the common carotid artery (CCA), and not extended to other segments. The prevalence of increased IMT and/or plaques is substantially higher in the carotid bulb or internal carotid artery, but the intended quantitative comparison between SSc patients and controls may be hindered by including these segments. An Acuson 128XP ultrasound device with a 7 MHz linear array transducer (Acuson Corporation, Mountain View, CA) was used for measuring IMT in all patients and controls, as described before.[7;20] With the subjects in supine position, the left common carotid arterial wall segment was scanned from a lateral transducer position and recorded on s-VHS tape. The common carotid artery wall segment was defined as 1 cm proximal to the carotid bifurcation. The far wall of the left common artery was assessed at three different positions. Off-line video analysis, using a previously described image analysis program,[21] was performed by one reader unaware of patient or control group data or characteristics. The highest IMT value found in this segment was considered to be the maximum IMT, the mean of three measurements in this segment was considered to be the mean IMT.

Blood analysis

Lipid levels were measured by routine techniques.

Additional serum and plasma samples for determination of markers of endothelial activation were stored at -20ºC until analysis. Serum levels of vascular cell adhesion molecule-1 (s-VCAM-1, R&D Systems, Abingdon, UK) and thrombomodulin (TM, Diaclone, Besançon, France) were measured according to the manufacturer's instruction. Serum was used to determine C-reactive protein and plasma was used to determine von Willebrand factor (vWF) using in-house enzyme linked immunosorbent assays (ELISA) as described before. [7]

Statistical analysis

Values are expressed as mean ± SD when variables were normally distributed, and as median with interquartile range (IQR, 25^{th} to 75^{th} percentile) in case of a non-normal distribution. Because the number of patients with diffuse cutaneous SSc (dSSc) was low in our patient group, subset analysis could not be performed. Differences between patients and controls were assessed by Student's t-test, by Mann-Whitney-U, and by X^2 (Pearson's chi-square or Fisher's exact test) as appropriate. Linear regression was used to assess the relationship between IMT and the different groups (SSc patients and healthy controls). An unadjusted analysis in which no corrections were made for possible confounders, and an adjusted analysis in which corrections were made for possible confounders are presented. The variables were manually entered. The level of significance for the association between group and outcome variable was set at $p < 0.05$. The variables age, sex, BMI and smoking were also studied as potential effect modifiers in the relationship of interest. The correlation between maximum IMT and disease related factors and endothelial markers was assessed by Spearman rank correlation coefficient since maximum IMT was non-normally

distributed. All analyses were carried out with the Statistical Package of Social Science, version 12.1. for Windows (SPSS, Inc, Chicago, IL)

RESULTS

Characteristics of patients and controls

The demographic characteristics and the traditional risk factors of SSc patients and healthy controls are shown in table 1. Patients tended to be older (55.4 ± 11.6 vs 50.9 ± 10.1 years, p=0.078) and used significantly more statins (14% vs 0%, p=0.038) than healthy controls. Patients had lower levels of HDL-cholesterol (1.40 (IQR 1.23-1.80) vs 1.68 (IQR 1.48-1.89) mmol/l, p=0.027) and higher levels of triglycerides (1.36 (IQR 1.16-2.14) vs 1.17 (IQR 0.77-1.67) mmol/l, p=0.030) than controls. No significant differences were found in other cardiovascular risk factors. Four patients had a history of macrovascular events compared with none in the control group.

Patients with SSc had a median disease duration of 6 years and had experienced Raynaud's phenomenon (RP) for almost 11 years. Limited cutaneous SSc was present in 92% and diffuse cutaneous SSc in 8% of patients. Patients had a median modified Rodnan Skin Score (mRSS) of 7.0 (IQR 4.5-14.0), a preliminary EScSG disease activity index of 0.5 (IQR 0.5-1.75) and a revised preliminary SSc severity scale score (Medsger's severity score, MSS) of 6.0 (IQR 4.5-7.0). The preliminary EScSG disease activity index may have been an underestimation of reality since not all variables (especially ESR and complement) were available. As shown in table 1, CRP levels were not substantially elevated, suggesting normal ESR levels. However, patients had significantly higher CRP levels than controls (3.5 (IQR 1.6-7.0) vs 0.8 (IQR 0.3-2.0), p<0.001). Forty-three percent of patients were current or former users of prednisolone with a median cumulative dose of 3.6 (1.9-16.1) grams (table 2).

Table 1 Traditional risk factors in SSc patients and healthy controls*

Characteristic	Patients (n=49)	Controls (n=32)	P value
Age (year)	55.4 ± 11.6	50.9 ± 10.1	0.078
Male sex (n (%))	8 (16%)	3 (9.4%)	0.513
Body mass index (kg/m^2)	23.7 ± 2.9	24.0 ± 2.9	0.582
Smoking (n (%))	3 (6%)	2 (6.5%)	1.000
Pack years for smokers (median (range))	20 (1-48)	10 (6-14)	
Diabetes mellitus (n (%))	2 (4%)	0	0.523
Hypertension, treated with antihypertensive agents (n (%))	12 (24%)	2 (6%)	0.120
Blood pressure (mm Hg)			
Systolic	120 (110-135)	120 (110-125)	0.409
Diastolic	75 (70-80)	75 (70-80)	0.379
Cholesterol			
Total (mmol/l)	5.22 ± 1.00	5.53 ± 1.06	0.217
LDL (mmol/l)	3.02 ± 0.85	3.06 ± 1.08	0.871
HDL (mmol/l)	1.40 (1.23-1.80)	1.68 (1.48-1.89)	0.027
Triglycerides (mmol/l)	1.36 (1.16-2.14)	1.17 (0.77-1.67)	0.030
Lipid-lowering drugs (n (%))	7 (14%)	0	0.038
Simvastatin (n, mean dose (mg))	4 (20)	0	
Atorvastatin (n, mean dose (mg))	2 (25)	0	
Rosuvastatin (n, mean dose (mg))	1 (10)	0	
Family history of CVD (n (%))	10 (20%)	5 (16%)	0.603
Cardiovascular history	4 (8%)	0	0.149
Cardiovascular	1		
Cerebrovascular	1		
Peripheral vascular disease	2		
CRP (mg/l)	3.5 (1.6-7.0)	0.8 (0.3-2.0)	<0.001

* Unless stated otherwise, data are expressed as mean ± SD when normally distributed and as median (interquartile ranges) when non-normally distributed

Abbreviations: LDL, low density lipoprotein; HDL, high density lipoprotein; CVD, cardiovascular disease; CRP, C-reactive protein

Table 2 Characteristics of patients with systemic sclerosis*

Disease characteristic	N=49
SSc subset (n (%))	
dcSSc	4 (8)
lcSSc	45 (92)
Disease duration (yr)	6 (2-12)
RP duration (yr)	11 (6-25)
Antibody (n (%))	
Scl70	4 (8)
centromere	22 (45)
nRNP	2 (4)
ANA, not specified	17 (35)
none	4 (8)
EScSG disease activity index	0.5 (0.5-1.5)
MSS	6.0 (4.5-7.0)
mRSS	7.0 (4.5-14.0)
Prednisolone use (n (%))	
None	28 (57)
Former	13 (27)
Current	8 (16)
Cumulative prednisolone dose (g)	3.6 (1.9-16.1)
Immunosuppresive agents (n (%))	
Never used	25 (51)
Former or current users	24 (49)
Methotrexate	
Current	12
Former	7
Cyclophosphamide	
Current	2
Former	4
Azathioprine	
Current	3
Former	3
Cyclosporin	
Current	0
Former	1

* Unless stated otherwise, data are expressed as mean ± SD when normally distributed and as median (interquartile ranges) when non-normally distributed.

Abbreviations: dcSSc: diffuse cutaneous systemic sclerosis; lcSSc: limited cutaneous systemic sclerosis; RP: Raynaud's phenomenon; Scl70: topoisomerase 1; nRNP: nuclear ribonucleoprotein; ANA: antinuclear antibodies; EScSG: European Scleroderma Study Group; MSS: Medsger's severity scale; mRSS: modified Rodnan skin score

Intima-media thickness

The median value for the mean IMT measurements in the common carotid artery was 0.69 mm (IQR 0.62-0.79) in patients and 0.68 mm (IQR 0.56-0.75) in controls (figure 1a). Also, IMT values were comparable between SSc patients and controls for each age decade. Amongst the 4 outliers in the group of SSc patients with IMT > 1.10 mm, one patient had a history of a cerebrovascular accident and one patient was known with left ventricular dysfunction probably caused by coronary artery disease. Both had other cardiovascular risk factors like current or former smoking and hypertension. The other 2 patients were not known with clinically manifest cardiovascular diseases. One of these patients had a family history of cardiovascular disease. Other risk factors were not present. Although plaques were not a primary endpoint, they were observed in 3 patients and not in healthy controls. Linear regression analysis of the mean IMT in the common carotid artery between controls and patients, when not corrected for possible confounders, demonstrated no significant difference in mean IMT (B=0.101, p=0.067) (table 3). Also no significant differences were seen between the groups after correction for the strongest confounders. Both in the model with the confounders age, HDL-cholesterol, and LDL-cholesterol (B=0.042, p=0.328) and in the model with the confounders age, HDL-cholesterol and history of macrovascular disease (B=0.030, p=0.474) no differences were found between patients and healthy controls. The possible relationship between mean IMT and SSc patients versus controls was lost when traditional risk factors were entered into the regression analysis. Addition of the confounder history of hypertension did not change the outcome. No correction for systolic and diastolic blood pressure, use of statins, smoking, diabetes mellitus, gender, BMI, total cholesterol or triglycerides was necessary since these outcome parameters were no confounders in the linear regression model. No effect modification by age, gender, BMI or smoking was found.

No significant differences were seen for the maximum IMT of the common carotid artery between SSc patients (0.83 mm, IQR 0.70-0.97) and healthy controls (0.77 mm, IQR 0.70-0.88) by means of linear regression analysis before and after correction for the confounders age, HDL-cholesterol, gender and history of macrovascular disease (see figure 1b, table 4). Addition of the other confounders, that is history of hypertension, diastolic blood pressure and LDL-cholesterol, did not change the outcome.

In patients no correlations were found between maximum IMT, CRP and disease related variables.

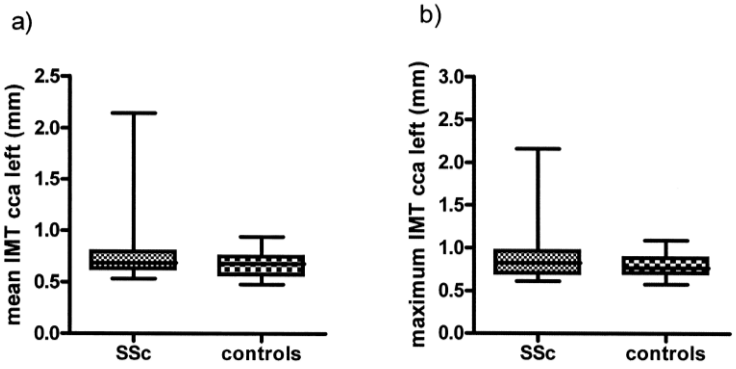

Figure 1 Box plots of (a) mean and (b) maximum left common artery intima-media thickness (IMT) of SSc patients and healthy controls. Data are uncorrected for confounders. The median, interquartile range, minimum and maximum values are shown.

Table 3. Linear regression analysis for risk factors for mean IMT of the left common carotid artery in SSc patients and healthy controls

Group (controls, patients)	B[#]	95% confidence interval	p-value
Unadjusted/crude	0.101	-0.007; 0.209	0.067
Adjusted*	0.042	-0.043; 0.128	0.328
Adjusted**	0.030	-0.054; 0.114	0.474

In the adjusted models corrections were made for the confounders * age, HDL-cholesterol and LDL cholesterol or ** age, HDL-cholesterol and history of macrovascular disease (see text); a total of 66 patients and controls were available for this analysis

[#] B is the regression coefficient

Table 4. Linear regression analysis unadjusted and adjusted for risk factors for maximum intima-media thickness of the left common carotid artery in SSc patients and healthy controls

Group (controls, patients)	B[#]	95% confidence interval	p-value
Unadjusted/crude	0.101	-0.017;0.219	0.093
Adjusted*	0.028	-0.071;0.127	0.577
Adjusted**	0.022	-0.081;0.126	0.668

* In the adjusted models corrections were made for the confounders * age, HDL-cholesterol and gender or ** age, HDL-cholesterol and history of macrovascular disease; a total of 66 patients and controls were available for this analysis

[#] B is the regression coefficient

Endothelial activation markers

Markers of endothelial activation were not increased in patients with SSc. Compared to controls, levels of VCAM-1 were even decreased (229 (IQR 188-311) ng/ml vs 287 (IQR 236-350) ng/ml, p= 0.014). No differences between SSc patients and controls were found in levels of vWF (72 (IQR 34-125)% vs 71 (IQR 48-110)%, p=0.691) and TM (3.8 (IQR 2.3-5.0) ng/ml vs 2.9 (IQR 2.1-3.7) ng/ml, p=0.151) (Figure 2). Levels of endothelial markers were not correlated with maximum IMT.

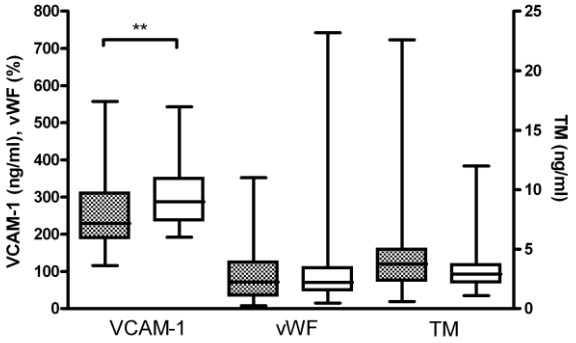

Figure 2 Endothelial activation markers. Boxes indicate the median value and the interquartile ranges. Lines indicate the minumum and maximum values. Dotted bars represent SSc patients, open bars controls. Abbreviations: VCAM-1: vascular cell adhesion molecule-1; vWF: von Willebrand factor; TM: thrombomodulin. ** $p<0.05$.

DISCUSSION

This study did not show differences in IMT of the common carotid artery and prevalence of plaques between patients with SSc and healthy controls, suggesting no increased prevalence of early atherosclerotic macrovascular disease in SSc Also, no correlations were found between IMT, disease related factors and markers of endothelial activation. Traditional risk factors, like increasing age and dyslipidaemia, accounted for increased IMT values in SSc patients and controls.

After the first reports suggesting an increased prevalence of macrovascular involvement in SSc, several studies have been performed the last decade using IMT of the carotid artery as a marker of early atherosclerosis. Lekakis et al,[22] Kaloudi et al,[23] and Bartoli et al[24;25] found strongly increased IMT values in the CCA in SSc patients compared with controls. In these studies mean IMT values were markedly higher than in our patients, while mean age was comparable. It is not known whether patient groups in studies by Kaloudi et al, [23] and Bartoli et al[24;25] are from overlapping cohort since these studies were performed in the same center and published in the same period. A larger percentage of dcSSc subtype was present in these studies compared with our study, although Kaloudi et al found no significant differences between mean IMT between subtypes.[23] On the other hand, our results are in agreement with Cheng et al[26;27] and Szucs et al,[28] who found no differences in IMT values in SSc patients compared with controls. Apart from a younger age and a larger percentage of dcSSc subtype in the study by Cheng et al,[26] age was comparable, as were IMT values. No difference was present in IMT values between subsets in this study either.[27] In view of this findings and given the small number of patients with dcSSc in our study, we did not perform a subset analysis. Overall, these discrepancies between studies in the presence of early atherosclerosis as measured by common carotid IMT in SSc patients might be explained by methodological differences, such as patients included in the study and comorbidity.

In our SSc patients lipid levels and statin use were statistically different from healthy controls. After correction for the strongest confounders in our model, no differences were seen in IMT values between SSc patients and healthy controls. Although statin use was no confounder in our model, a meta-analysis showed that statin therapy is efficient to decrease the rate of carotid atherosclerosis progression in the long-term.[29] Otherwise, statins may have a potential benefit in preventing endothelial dysfunction in SSc patients.[30]

Treatment with immunosuppressive agents, especially corticosteroids , influences the atherogenic process. Corticosteroids are considered to have atherogenic properties,[31] like azathioprine,[32] whereas for hydroxychloroquine[31] and methotrexate[9] a protective effect against atherosclerosis has been described. Otherwise, immunosuppressive therapy with prednisolone, cyclophosphamide or hydroxychloquine was associated with the absence of plaques in patients with sytemic lupus erythematosus.[33] It is difficult to establish whether the observed associations between immunosuppressive agents and atherosclerosis are due to the immunosuppressive agents themselves, or their effect on the activity of the autoimmune disease. In our study 49% were former or current users of immunosuppressive agents. No association was found between maximum IMT and cumulative prednisolone dose and use of other immunosuppressive agents.

Markers of inflammation, such as C-reactive protein, are related to the risk of cardiovascular and peripheral vascular disease. Increased levels of CRP are associated with increased risk of symptomatic disease.[34;35] In our population, CRP levels were significantly elevated compared to healthy controls. The CRP levels we found might have been associated with future coronary events,[34;35] but we found no association between CRP and IMT values. This can be explained by the study design. Our study was not designed to find a relationship between CRP and risk of cardiovascular disease, and we did not exclude other conditions that could explain elevated CRP levels, like intercurrent infections. Otherwise, in SSc patients, besides elevations due to infection, no significant elevations of CRP levels are seen.[36]

Surprisingly, we did not find elevated levels of endothelial activation markers. Our population of SSc patients was heterogeneous with respect to disease duration. The average patient had inactive disease. Most inflammation is expected in the early stages of the disease or in patients with active disease. Also, by using Medsger's severity index we could not find an association between macrovascular disease and the severity of SSc. This might explain the absence of increased levels of endothelial activation markers. All these data point to absence of premature atherosclerosis in SSc.

Our results might be an underestimation of atherosclerosis in SSc patients and controls. Besides the possible explanations as stated above, our patients predominantly were suffering from lcSSc, in which inflammation is not always present.[37] However, when analyzed in subsets, other authors did not find differences in IMT values between subsets.[23;27] Furthermore, we used IMT values of the CCA. This segment is commonly evaluated in our laboratory as it can be approached easily and measurements on this segment are reproducible. Using the same protocol as described here, we found increased IMT values in SLE patients.[38] However, atherosclerotic lesions appear later in the CCA than in the internal carotid artery (ICA) or bulb, but these latter two segments are more difficult to visualize.[39] Also, it can be difficult to assess whether IMT of the CCA represents atherosclerosis or vascular hypertrophy.[40] Although other non-invasive markers

of early changes in the arterial wall are available, such as arterial wall thickening and stiffening, carotid IMT has been used more frequently, and has been found to be a strong predictor of future vascular events.[40;41]

CONCLUSION

IMT of the CCA is not increased in patients with SSc compared to controls, both when uncorrected and corrected for traditional risk factors. So, SSc seems not associated with increased early atherosclerotic macrovascular disease. Although SSc is characterized by endothelial dysfunction, it seems that this is mainly reflected in microvascular disease.

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CHAPTER 8

SKIN AUTOFLUORESCENCE, AS A MARKER OF ACCUMULATION OF ADVANCED GLYCATION ENDPRODUCTS AND OF CUMULATIVE STRESS, IS NOT INCREASED IN PATIENTS WITH LIMITED CUTANEOUS SYSTEMIC SCLEROSIS

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ABSTRACT

Objective: To investigate whether advanced glycation endproducts (AGEs) in the skin are increased in patients with limited cutaneous systemic sclerosis (lcSSc), and are related to the presence of disease-related and traditional cardiovascular risk factors.
Methods: Skin autofluorescence, as a measure for the accumulation of AGEs, was assessed by measuring UV-A light excitation-emission matrices (AF-EEMS) in 41 SSc patients and 41 age- and sex-matched controls. Traditional cardiovascular risk factors and disease-related risk factors were recorded.
Results: Skin AF-EEMS did not differ between SSc patients and controls (1.68 ± 0.58 a.u. vs 1.63 ± 0.41 a.u., p=0.684). Skin AF-EEMS in SSc patients was associated with levels of CRP (r=0.44, p=0.004), Medsger's severity scale (r=0.45, p=0.006), and use of agents intervening in the renin-angiotensin system (r=0.33, p=0.027). When analysing SSc patients and controls together, in multivariate analysis, only age and use of agents intervening in the renin-angiotensin system were independently associated with AF-EEMS.
Conclusion: These data demonstrate that skin AGEs are not increased in lcSSc patients.

INTRODUCTION

Vascular involvement is a key factor in major manifestations of systemic sclerosis (SSc), such as Raynaud's phenomenon (RP), myocardial dysfunction, pulmonary hypertension and renal involvement. Microvascular involvement, in which endothelial injury is present, is the main characteristic of SSc.[1;2] Oxidative stress has been suggested as a major player in the process of endothelial dysfunction found in SSc. Endothelial damage may be induced by oxygen free radicals and reactive nitrogen species, generated locally by the inflammatory process and by periods of tissue ischemia followed by post-ischaemic reperfusion.This so-called ischaemic-reperfusion injury can be seen in RP.[3;4] Increased levels of antibodies against oxidised low density lipoproteins (LDL)[4-6] and increased serum levels of 8-isoprostane,[7] being markers of oxidative stress, have, indeed, been observed in SSc.

Oxidative or carbonyl stress, leading to formation of so-called reactive carbonyl compounds, is an important source for the generation of so-called advanced glycation endproducts (AGEs).[8] AGE generation as a result of oxidative stress has also been found in inflammatory diseases, such as rheumatoid arthritis and SLE.[9-15]

Tissue autofluorescence (AF) is a marker of the accumulation of AGEs, validated in different patient groups and healthy controls.[16-18] Therefore, we assessed AGE accumulation in patients with SSc, and hypothesized that AGE accumulation is increased in patients with SSc compared to healthy controls based on the presence of oxidative stress and endothelial dysfunction in SSc. We related AGE accumulation to the presence of disease-related and traditional cardiovascular risk factors.

PATIENTS AND METHODS

Patients

Forty-one patients with limited cutaneous SSc from our university medical center out-patient clinic, fullfilling the ACR criteria for SSc,[19] were included. Exclusion criterium was pregancy. Forty-one age and sex- matched healthy subjects were recruited as controls. The local research ethics committee gave approval for the study, and informed consent was obtained from all subjects. Clinical data were obtained by chart review and questionnaires. Diabetes mellitus was defined by the criteria from the American Diabetes Association. Dyslipidemia was diagnosed if plasma cholesterol exceeded 6.21 mmol/l, LDL-cholesterol exceeded 3.36 mmol/l, triglycerides exceeded 2.26 mmol/l, or when the patient used lipid-lowering drugs.[20] Hypertension could not be categorized because of frequent use in SSc patients of vasodilating agents, such as calcium channel antagonists and ketanserin for other reasons than hypertension. Creatinine clearance (CrCl) was estimated using the Cockcroft-Gault formula. Smoking status and body mass index were also recorded. The SCORE risk estimation system was used, which was originally developed to obtain an estimation of total 10 years fatal cardiovascular risk in populations, using gender, age, total cholesterol level, systolic blood pressure and smoking status.[21] Furthermore, we assessed disease-related factors that might influence skin AF and the development of atherosclerosis. Modified Rodnan Skin Score was assessed to determine skin thickness. To assess disease activity the

preliminary European Scleroderma Study Group (EScSG) activity indices (a score ranging from 0-10) was used. A score higher than 3 denotes active disease.[22;23] Also, the revised preliminary SSc severity scale (Medsger's severity scale), a measure of activity, damage and severity, was used. This scale is a 9-organ disease severity scale in which for each organ system a score of 0 to 4 is applied, with 0 being normal and 4 denoting endstage organ involvement.[24] Immunosuppressive therapy was recorded.

Assessment of skin AF

Tissue AGEs accumulation can be assessed as skin autofluorescence (AF), following the priciples of the AGE Reader, which is a validated and non-invasive technique.[16;25] Repeated measurements on one day in controls and diabetic patients showed an overall Alman error percentage of 5%. In this study, an adapted set-up of the AGE Reader was used, namely the Excitation Emission Matrix Scanner (EEMS), which is a technique to determine skin autofluorescence (AF-EEMS), which has the additional potential to discriminate between autofluorescence spectra from different fluorophores. This technique and set-up has been described.[26] Measurement was performed at a skin site of approximately 4 cm ^{2} without evidence of fibrosis at the ventral site of the forearm, or other skin lesions. A series of measurements was obtained for each subject and mean skin AF-EEMS was determined as described.[14] Skin pigmentation is also known to influence autofluorescence by light absorption. Therefore skin reflection should be >10% to perform an adequate measurement.

Laboratory assessments

Lipid concentrations (total, high density lipoprotein (HDL) and low density lipoprotein (LDL) cholesterol and triglycerides), glucose and creatinin were measured by routine techniques. CRP was measured using in-house enzyme-linked immunosorbent assays (ELISAs) as described.[27]

Statistical analysis

Values are expressed as mean ± SD when variables were normally distributed. In case of a non-normal distribution, values are reported as median (interquartile ranges). For comparison between groups, continuous variables were analysed by Student's t-test or Mann-Whitney U tests, as appropriate. In case of categorical variables, the chi-square test was used. The univariate correlation between AF-EEMS values and other categorical variables was assessed by Pearson correlation coefficient in case of normal distribution. Otherwise, Spearman correlation coefficient was used. To assess the influence of tested parameters multiple regression analysis using the backward method was performed to assess the influence of demographic variables, outcome variables and disease related factors on skin autofluorescence (AF-EEMS). Variables which were significantly correlated in univariate analysis were used as independent variables in the multivariate analysis.
All analyses were performed using SPSS 14.0. A two-tailed p-value < 0.05 was considered statistically significant.

Table 1. Clinical characteristics

	Patients (n=41)	Controls (n=41)	P-values
Age (years)	55.9 ± 11.0	55.4 ± 9.0	NS
Female, n (%)	33 (80%)	33 (80%)	NS
History of CVD, n (%)	4 (9%)	0	NS
Family history of CVD, n (%)	9 (22%)	13 (32%)	NS
Diabetes mellitus, n (%)	2 (5%)	0	NS
Glucose (mmol/l)	4.9 (4.3-5.4)	5.6 (5.3-6.2)	<0.001
BMI (kg/m^2)	24.1 (21.1-25.3)	25.0 (22.2-27.1)	0.019
Creatinine (µmol/l)	80 (67-93)	77 (59-84)	NS
Creatinine clearance (ml/min/1.73 m^2)	78 ± 23	87 ± 14	NS
Current smoking	3 (7%)	0	NS
Blood pressure			
Systolic (mm Hg)	120 (110-138)	128 (118-140)	NS
Diastolic (mm Hg)	75 (70-80)	78 (72-87)	0.023
Antihypertensive or vasodilating agents	33 (80%)	1 (2%)	<0.001
ACE inhibitors or ATII receptor blockers	14 (34)	1 (2%)	<0.001
Lipid levels			
Cholesterol (mmol/l)	5.0 ± 0.9	5.8 ± 0.9	<0.001
HDL-cholesterol (mmol/l)	1.3 (1.2-2.2)	1.7(1.4-2.0)	0.025
LDL-cholesterol (mmol/l)	2.9 ± 0.8	3.5 ± 0.8	0.001
Triglycerides (mmol/l)	1.4 (1.2-2.2)	1.2 (0.9-1.7)	0.035
Statin use	7 (17%)	0	0.012
Dyslipidemia, n (%)	18 (44)	19 (46)	NS
Aspirin use	12 (29%)	0	<0.001
SCORE, %	1.0 (0.0-2.0)	1.0 (0.0-2.0)	NS
CRP (mg/l)	3.4 (1.6–7.7)	1.5 (0.6-2.5)	<0.001

Unless stated otherwise, data are expressed as mean ± SD when normally distributed, and as median (25-75%) when non-normally distributed.

CVD, cardiovascular disease; BMI, body mass index; ACE, angiotensin converting enzyme; ATII, angiotensin II; HDL, high density lipoprotein; LDL, low density lipoprotein; SCORE, systematic coronary risk evaluation; CRP, C-reactive protein

RESULTS

Characteristics of patients and controls

Characteristics of patients and controls are presented in table 1 and 2. Patients and controls were similar in age and gender and regarding (family) history of CVD, presence of diabetes mellitus and renal function. Significant differences between SSc patients and controls were present in diastolic blood pressure and lipid levels, but also in medication used. Antihypertensive agents or vasodilating agents were used in 80% of patients compared to 2% of controls. Amongst these agents, 11 patients used angiotensin converting enzyme (ACE) inhibitors and 3 patients used an angiotensin II receptor blocker. Also, statins were used more frequently in patients. BMI was slightly higher in controls, and none of the controls had a history of cardiovascular disease. Although the presence of traditional risk

factors differed significantly between patients and controls, the total cardiovascular risk, SCORE, was comparable. CRP levels were significantly increased in patients compared to controls (3.4 mg/l (IQR 1.7-7.7) vs 1.4 mg/l (0.6-2.5), p<0.001). Immunosuppressive agents, such as prednisolone, methotrexate, azathioprine and cyclophosphamide were used in 15 (37%) SSc patients

Table 2. Disease characteristics and disease related factors in SSc patients

Characteristic	Patients, n (%)
Duration SSc, yr	5 (3-11)
Duration RP, yr	11 (5-23)
EScSG activity index	0.5 (0.5-2.0)
MSS	6 (5-7)
mRSS	7 (5-14)

Data are expressed as median (interquartile ranges)

SSc, systemic sclerosis; RP, Raynaud's phenomenon; EscSG: European Scleroderma Study Group; MSS, Medsger's severity scale; mRSS, modified Rodnan Skin Score

Skin AF-EEMS in patients and controls

No difference was found in AF-EEMS between patients and healthy controls (1.68 ± 0.58 a.u. vs 1.63 ± 0.41 a.u., p=0.684, figure 1). AF-EEMS was significantly higher in those with a history of CVD (n=4) compared to those without a history of manifest CVD (n=77) (2.22 ± 0.81 a.u.vs 1.62 ± 0.47 a.u., p=0.020). In subjects using ACE inhibitors or ATII receptor blockers for hypertension or other reasons, AF-EEMS was significantly higher than in subjects not using these agents (2.01 ± 0.62 a.u. vs 1.57 ± 0.45 a.u., p=0.002).

Univariate analysis between skin AF-EEMS and traditional risk factors and disease related factors for CVD in SSc patients resulted in a positive correlation between skin AF-EEMS and CRP (r=0.44, p=0.004), as well as MSS (r=0.45, p=0.006) and use of ACE inhibitors or ATII receptor blockers (r=0.33, p=0.027). Univariate analysis of all subjects, patients and controls together, resulted in an association between skin AF-EEMS and age (r=0.28, p=0.010), CRP (p=0.25, p=0.026), and use of ACE inhibitors or ATII receptor blockers (r=0.275, p=0.013). All other clinical and biochemical variables did not show significant correlations with skin AF-EEMS.

Multivariate analysis revealed that age and use of ACE inhibitors or ATII receptor blockers were independently associated with skin AF-EEMS. Otherwise, no independent associations with skin AF-EEMS were present (table 3).

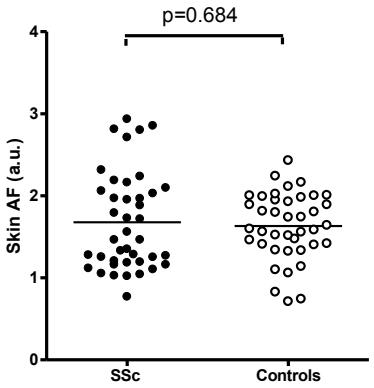

Figure 1. Skin autofluorescence (AF-EEMS) in patients (closed circles) and controls (open circles). The horizontal line represents mean skin AF-EEMS values.

Table 3. Multiple linear regression analysis with AF-EEMS as dependent variable in patients with systemic sclerosis and healthy controls (n=82).

	B	β	P-values
Constant	0.284		
Age	0.022	.400	0.006
Use of ACE inhibitors or ATII receptor blockers	0.613	.494	0.001

AF-EEMS, autofluorescence obtained by the Excitation-Emission Matrix Scanner; ACE, angiotensin converting enzyme; ATII, angiotensin II

DISCUSSION

In this study, we demonstrated that skin AF as a marker of tissue AGE accumulation is not increased in SSc patients, while expected relations with age, prevalence of CVD and CRP were found.

To our knowledge, only one study has been performed on the relation between AGEs and SSc. Kaloudi et al [28] compared circulating levels of Nε-(carboxymethyl)lysine (CML), one of the AGEs which can be detected in vivo, in SSc patients and healthy controls by means of ELISA. They found increased CML levels in SSc patients regardless of subset, with highest levels found in SSc patients with an 'early' disease pattern in nailfold videocapillaroscopy, suggesting that AGEs are involved in SSc microangiopathy. This is in agreement with the observation that highest values of markers of oxidative stress are seen in early stages of the disease.[4]

Several factors may be responsible for this discrepancy. We used noninvasive skin autofluorescence measurements for the assessment of accumulation of AGEs. This technique is simple, rapid and non-invasive. Results from this technique were found to correlate strongly with levels of AGEs measured from skin biopsies,[16;29] although this validation was not extended to patients with SSc. Also, AGE detection in tissue with long-

lived (years) proteins like the skin, may better reflect the chronic accumulation of AGEs than measuring AGEs from serum or plasma with a relatively short (weeks) half-life of most proteins.[25] Since disease activity in SSc patients will wax and wane, we considered noninvasive skin autofluorescence measurements more useful in these patients. Our choice for the skin autofluorescence was also supported by the increased levels found in other autoimmune diseases with intermittent disease activity like rheumatoid artitis and SLE in which skin AF was related to integrated disease duration and damage.[14;15] Another consideration might be that skin AF was affected in the same emission range by other fluorophores (like NADH or tryptophan) to a different extent than in other conditions. Although we cannot exclude this, the expected relations with factors like age, CRP, and history of CVD still supports skin AF as a marker of AGEs.

We expected to find more AGE accumulation in SSc patients with signs of inflammation and disease severity. Compared to controls, we found higher CRP levels in SSc patients, which might suggest a more active disease although absolute levels were not increased and EscSG activity index did not reflect active disease. We found a positive correlation between skin AF-EEMs levels and MSS, a measure of activity, severity and damage in SSc patients, suggesting a relation between AGE accumulation and disease severity. Recently, doubts have been raised by Valentini et al. on the weighting of the contribution of the different organ systems in the MSS.[30] Perhaps such a disbalance in the MSS explains why MSS, but also CRP, were not found to remain as predictors of skin AF-EEMS in multivariate analysis.

We found higher skin AF-EEMS levels in a substantial number of subjects using ACE inhibitors or ATII antagonists compared to subject not using these agents, and their use was independently associated with skin AF-EEMS in multivariate analysis. This seems surprising because these agents have been found to reduce AGE accumulation in animal studies and in vitro studies.[31-35] In our SSc patients, these agents were used for a long period for several reasons, such as hypertension and Raynaud's phenomenon. Therefore, this treatment would have been expected to diminish AGE accumulation. We found higher skin AF-EEMS levels in subjects using these agents, but it cannot be ruled out that AGE accumulation was even more pronounced before agents intervening in the renin-angiotensin system were used.

Skin fibrosis in SSc could be a possible explanation of the lack of increase in AF-EEMS values, that we had expected to occur, although we only included patients with limited cutaneous SSc, who had no significant fibrosis of the forearm. AGE accumulation and skin autofluorescence are strongly related to collagen-linked fluorescence and, thereby, to the usually very long half-time (15-20 years) of collagen. Previously reported increases in both collagen synthesis but especially increased degradation of skin collagen in SSc suggest that our results in SSc patients may be explained by accelerated skin collagen turnover.[36-40] In that case, the accelerated degradation of skin collagen could have prevented skin AGE accumulation, and masked the effects of oxidative stress on AGE formation. Although assessment of skin AF-EEMS was performed at visible and palpable non-lesional skin to prevent influences by the presence of skin fibrosis, this influence cannot be ruled out completely. Also, increased collagen turnover may not be limited to affected skin only.

In conclusion, although oxidative stress seems present in SSc and is an acknowledged important factor in the generation of AGEs, increased levels of AGEs, as

determined by skin autofluorescence were not found. We cannot rule out that accumulation of AGEs in SSc patients was prevented by the use of ACE inhibitors or ATII receptor blockers, and by accelerated skin collagen turnover in clinically unaffacted areas.

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CHAPTER 9

SUMMARY AND FUTURE PERSPECTIVES

SUMMARY

In this thesis studies are presented concerning vascular involvement in systemic sclerosis (SSc). Besides microvascular involvement and endothelial cell dysfunction, this thesis also focuses on macrovascular involvement and therapy of Raynaud's phenomenon (RP). A general introduction is given in **chapter** 1. The studies described in this thesis aim to contribute to insight into the mechanisms of vascular involvement in SSc, its consequences, and therapy. The studies are introduced in chapter 1.

Microvascular involvement and endothelial cell dysfunction

In **chapter 2 and 3** the results are presented of a single center open study, in which we assessed the effects of bosentan, an endothelin-1 receptor antagonist, in SSc patients with severe RP. In this study, 15 patients with limited cutaneous SSc were treated with bosentan for 16 weeks with a follow-up period of 4 weeks. The study started in winter 2005/2006 and use of bosentan ended in spring 2006. In **chapter 2** we describe the effects of bosentan on RP. Patients had to make a diary for recording frequency, duration and severity of RP attacks. Photoelectric plethysmography (PEP) during cooling and rewarming was performed at baseline, after 8 and 16 weeks, and at the end of the follow-up period. Treatment with bosentan resulted in an encouraging improvement in the frequency, duration and severity of RP attacks. Although seasonal temperature differences could have contributed to this result, no difference in outdoor temperature was seen until week 12, a time when significant subjective improvement was already noted. However, we could not demonstrate an objective improvement in blood flow, as measured by PEP. Also in other RP trials, an objective improvement in blood flow is not always found. Also intraindividual variations and a moderate reproducibility of PEP could have contributed to the lack of objective improvement. Since our study was not a placebo-controlled trial, a placebo-effect cannot be ruled out.

In **chapter 3** changes in endothelial cell dysfunction during treatment with bosentan were studied with assessment of vasodilatory microvascular responses using laser Doppler fluxmetry combined with iontophoresis, capillary permeability using fluorescence videomicroscopy, total number of capillary loops using nailfold microscopy, and markers of endothelial activation. We observed no significant changes in endothelial-dependent and endothelial-independent vasodilation. Also, capillary permeability and total number of nailfold capillaries remained unchanged. Levels of thrombomodulin decreased significantly, but no effect was noted on the levels of the endothelial activation marker vascular cell adhesion molecule-1. We concluded that 16 weeks treatment with bosentan did not result in structural improvement of endothelial damage. The major limitation of this study is its uncontrolled and open design with a relatively short duration. Another explanation might be the presence of irreversible structural changes, such as focal stenosis and occlusions in digital arteries, which are common manifestations in SSc patients.

Although other authors did not observe improvement in the severity of RP or did not investigate the effect of bosentan on RP, they confirmed our results of the effects of bosentan on digital vascular structure and function.[1;2]

Vascular or endothelial dysfunction has been suggested to be a crucial element in the pathogenesis of SSc. In **chapter 4** we describe the results of a case-control study regarding endothelial dysfunction. Forty-seven patients and twenty-three healthy controls were studied. Microvascular skin reactivity was assessed using laser Doppler fluxmetry combined with iontophoretic administration of acetylcholine (endothelium-dependent vasodilator) and sodium nitroprusside (endothelium-independent vasodilator). Both endothelium-dependent and endothelium-independent vasodilation were significantly decreased in patients with SSc. Our results are in agreement with previous studies. Since endothelial dysfunction has also been found in other diseases, such as hypertension, diabetes mellitus and obesity, we conducted a multiple regression analysis to relate vasodilation with the presence of these disorders and possible other confounding variables. This analysis revealed that the decrease in endothelium-dependent, but not endothelium-independent, vasodilation in SSc patients was related to the presence of SSc. The impaired endothelium-independent vasodilation was not statistically significant anymore after correction for confounders ($p=0.076$). Therefore, we cannot rule out that, besides endothelial dysfunction, structural defects or functional changes in the arteriolar wall of the microcirculation are present in SSc patients with a median disease duration of 7 years (IQR 3-12 years).

In SSc increased capillary permeability or leakage has been demonstrated using nailfold capillaroscopy. Besides increased capillary permeability, other nailfold capillary abnormalities are present in SSc, such as a reduced capillary density, bushy patterns, extravasates and giant capillaries. The increased capillary permeability of the nailfold capillaries is hypothesized to be a protective mechanism at least in the digits. As described in **chapter 5**, we used large-window videodensitometry with sodium fluorescein at the level of the ankle, in order to test the hypothesis that capillary leakage is a generalized phenomenon in SSc. Thirty-eight SSc patients were compared with 20 healthy control subjects. Transcapillary sodium fluorescein leakage was not increased in SSc patients, but significantly decreased in comparison with healthy controls. As also observed in the nailfolds, capillary density at the level of the ankle was decreased in SSc patients. In all subjects capillary leakage, expressed as the average relative fluorescence light intensity over the first 7 minutes ($I_{av}(7)$) of the videodensitometry, correlated positively with BMI and capillary density. In multivariate analysis, differences between SSc patients and healthy controls in capillary leakage ($I_{av}(7)$) were related to differences in capillary density, BMI and HDL cholesterol. This is the first study to document that decreased capillary density is present at the ankle in SSc patients confirming microvascular involvement. Also, a more unfavourable cardiovascular risc profile, i.e. increased BMI and lower HDL cholesterol levels, was found in controls. This might also explain higher levels of capillary permeability in controls compared to SSc patients. The decreased capillary permeability in SSc patients could be explained by the decreased capillary density, and therefore, we cannot confirm our hypothesis that capillary permeability is a generalized phenomenon in SSc at the level of individual capillaries.

CHAPTER 9

Macrovascular involvement

A review of the literature regarding the prevalence of macrovascular disease in SSc is presented in **chapter 6**. Several autoimmune rheumatic diseases are characterized by premature and accelerated atherosclerosis in which both classical and non-classical risk factors contribute to atherogenesis. SSc is characterized by widespread vascular involvement, and microvascular involvement, such as RP, is well-known. In contrast to other autoimmune rheumatic diseases, less attention has been paid to macrovascular involvement. As expected, an increased prevalence of distal peripheral artery disease in the digits has been found. Otherwise, no increased prevalence of coronary artery disease, cerebrovascular disease and peripheral arterial disease has been observed. Using markers of early atherosclerosis, such as intima-media thickness (IMT) of the common carotid artery, discrepant results have been reported. However, as in other autoimmune rheumatic diseases, non-classical risk factors, such as increased lipoprotein [a], oxidized LDL, inflammation, endothelial dysfunction, and markers of vascular damage are present in SSc.

In **chapter 7** we studied the prevalence of early signs of subclinical atherosclerosis, as assessed by IMT of the common carotid artery, in our cohort of SSc patients. We compared IMT of 49 SSc patients with 32 healthy controls. IMT did not differ significantly between patients and controls. Since patients tended to be older and had a different lipid profile than healthy controls, a linear regression analysis has been performed adjusted for confounders. Also, when adjusted for confounders, such as age and lipid profile, no difference in IMT was found between patients and controls. In SSc patients endothelial activation markers were not increased in comparison with controls. We did not find a correlation between IMT and disease related variables or markers of endothelial activation. As described in our review, we concluded that SSc is not associated with an increased prevalence of early signs of atherosclerosis.

It is generally known that SSc is characterized by microvascular involvement. Endothelial dysfunction of the microcirculation is present, and oxidative stress has been suggested to be a major player in this process. Markers of oxidative stress have been observed in SSc. Oxidative stress is an important step in the generation of advanced glycation endproducts (AGEs). The accumulation of AGEs is related to age, but occurs more rapidly in conditions like diabetes, renal failure and atherosclerosis. In other autoimmune inflammatory rheumatic diseases, as rheumatoid arthritis and systemic lupus erythematosus, acummulation of AGEs as a result of oxidative stress and inflammation has been observed. Based on the presence of oxidative stress and endothelial dysfunction in SSc, we hypothesized that AGE accumulation is increased in SSc patients. We used skin autofluorescence (AF) for the assessment of AGE accumulation. and related AGE accumulation to classical and non-classical (disease-related) risk factors for cardiovascular disease. In **chapter 8** skin AF by measuring UV-A light excitation emission matrices (EEMS) was assessed in 41 SSc patients and 41 age- and sex-matched healthy controls. Skin AF-EEMS did not differ between SSc patients and controls. Skin AF-EEMS in SSc patients was associated with CRP levels, Medsger's severity scale and the use of agents intervening in the renin-angiotensin system. In patients and controls together, skin AF-

EEMS was associated with age, as have been observed previously. In multivariate analysis, only age and use of agents intervening in the renin-angiotensin system were independently associated with AF-EEMS. Although oxidative stress and endothelial dysfunction are present in SSc, and despite similarities in pathogenic factors with other autoimmune inflammatory rheumatic diseases, no increased AF-EEMS levels were found in SSc patients, reflecting no increased accumulation of AGEs in skin. However, we cannot rule out that subclinical skin fibrosis and accelerated collagen degredation, and the use of ACE inhibitors or ATII receptor blockers have masked detection of increased skin autofluorescence by preventing AGE accumulation in SSc.

Conclusions of this thesis

1. Treatment with bosentan, an ET-1 receptor antagonist, resulted in encouraging improvement of frequency, duration and severity of RP attacks in an observational study in 15 patients with limited cutaneous SSc and severe RP.
2. Bosentan therapy did not result in significant improvement of endothelial dysfunction in these patients.
3. Microvascular involvement is a primary characteristic of SSc.
4. Increased capillary permeability at the level of individual capillaries in the nailfolds of SSc patients cannot be generalized. However, decreased capillary density at the ankle was found in SSc patients confirming generalized microvascular involvement.
5. Unlike other autoimmune inflammatory rheumatic diseases, no increased prevalence of clinically manifest or subclinical early atherosclerosis is apparent in SSc.
6. Skin autofluorescence is not increased in SSc, in contrast to RA and SLE, despite the presence of oxidative stress and endothelial dysfunction in SSc.

Future perspectives

Pathophysiology and therapy of SSc and SSc-associated RP remains a challenge. The same applies to the correct assessment of digital vascular structure and function. As described, none of the various techniques used in the assessment of vascular structure and function in patients with primary or secondary RP is ideal, since they all have limitations.[3] In the studies performed in this thesis, interpreting results of the vascular studies was hampered, amongst others, by the presence of skin fibrosis and the use of vasodilating agents. In general, a validated and standardized technique, also valuable for the quantitative assessment of peripheral vascular disease is missing.

A placebo-controlled trial is necessary to prove the efficacy of bosentan on RP in SSc patients, and on functional and structural abnormalities in the microvasculature in SSc associated RP. Such a trial is underway.

Microvascular disease is clearly present in SSc. To what extent this is structurally and functionally generalized has still to be established. Novel therapeutic agents are necessary for treating the vasculopathy in SSc. Statins, known for its lipid-lowering effects, have also promising effects on vascular function by protecting the endothelium and improving its functional activity, mobilization of endothelial precursors, suppression of the inflammatory

response, and inhibition of fibrosis.[4-7] Furthermore, the absence of increased prevalence of macrovascular disease, as seen in other autoimmune inflammatory rheumatic diseases, should be explained. This may shed light on possible presence of factors preventing the development of early atherosclerosis in autoimmune inflammatory diseases.

Accumulation of AGEs has been demonstrated to be involved in atherosclerosis and to predict cardiovascular disease in patients with diabetes and renal failure. Skin AF-EEMS is not validated in patients with SSc, and the influence of skin fibrosis is unknown. It would be worthwhile to investigate the validation of AF-EEMS for noninvasive assessment of AGEs in skin against AGE assays in skin biopsies and plasma levels of AGEs. Thereafter, it would be interesting to investigate longitudinally skin AF-EEMS levels as a marker of oxidative stress and endothelial dysfunction in SSc.

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NEDERLANDSE SAMENVATTING

(voor niet-ingewijden)

Sclerodermie

Sclerodermie of systemische sclerose is een reumatische aandoening waarbij het bindweefsel in de huid, maar ook elders in het lichaam steeds stugger wordt (*fibrosering)*. Sclerodermie betekent harde huid en is afkomstig van de Griekse woorden 'scleros' (hard) en 'derma' (huid).

Het is een zeldzame aandoening. Geschat wordt dat er in Nederland drieduizend mensen sclerodermie hebben, waarbij de diagnose jaarlijks bij ongeveer honderd personen wordt gesteld. Sclerodermie komt drie keer vaker voor bij vrouwen dan bij mannen, en de diagnose wordt meestal gesteld tussen het dertigste en vijftigste levensjaar.

De oorzaak van sclerodermie is onbekend. Duidelijk is dat naast de fibrosering beschadiging van de kleine bloedvaatjes een belangrijke rol speelt en dat ook ons eigen afweersysteem *(immuunsysteem)* betrokken is.

Er zijn twee vormen van sclerodermie: de lokale vorm, waarbij alleen de huid is aangedaan, en de systemische vorm, waarbij er ook sprake is van betrokkenheid van de inwendige organen.

De systemische sclerodermie kent 2 vormen, namelijk een gelimiteerde en een diffuse vorm. De gelimiteerde vorm *(limited cutaneous systemic sclerosis)* kenmerkt zich door het feit dat de huidafwijkingen zich beperken tot de onderarmen, onderbenen en het gezicht. Bij de diffuse vorm *(diffuse cutaneous systemic sclerosis)* strekken de huidafwijkingen zich ook uit over de bovenarmen, bovenbenen en romp. Bij beide vormen kan er betrokkenheid zijn van de inwendige organen.

Het natuurlijk beloop is erg wisselend, maar kan leiden tot een kortere levensverwachting afhankelijk van de aantasting van de inwendige organen. Overlijden ten gevolge van sclerodermie is met name het gevolg van longproblemen, waarbij de longen door toegenomen bindweefsel *(longfibrose)* slechter gaan werken, of van een verhoogde bloeddruk in de longslagaders *(pulmonale hypertensie)*.

De kwaliteit van leven wordt bepaald door o.a. de mate van huidbetrokkenheid, orgaanbetrokkenheid en vermoeidheid. Eén van de belangrijkste verschijnselen die van invloed zijn op het algemeen welbevinden is de aanwezigheid van het fenomeen van Raynaud en de aanwezigheid van zweertjes aan de vingertoppen *(digitale ulcera)*.

Fenomeen van Raynaud

Het fenomeen van Raynaud is genoemd naar een Franse arts, Maurice Raynaud, die de verschijnselen die naar hem genoemd zijn voor het eerst beschreef. Het fenomeen van Raynaud kenmerkt zich door terugkerende perioden waarbij de bloedvaten in de vingers of tenen zich vernauwen als reactie op kou of een spannende gebeurtenis. Er treedt een verkleuring van de vingers op die in drie fasen verloopt: eerst wit, vervolgens paarsblauw en rood. Deze klachten kunnen gepaard gaan met pijn en stijfheid. Het fenomeen komt voor bij gezonde mensen als een opzichzelf staand verschijnsel, en wordt dan primair fenomeen van Raynaud genoemd. Wanneer er sprake is van een onderliggend reumatisch ziektebeeld, bijv sclerodermie, spreken we van een secundair fenomeen van Raynaud. Bij patiënten met sclerodermie heeft >90% verschijnselen van het fenomeen van Raynaud. De terugkerende perioden met bloedvatvernauwing (Raynaud aanvallen) en de ernst hiervan beïnvloeden de kwaliteit van leven van patiënten met sclerodermie. In ernstige gevallen kunnen er door de verminderde doorbloeding van de vingers of tenen zweren ontstaan, en kunnen de vingers of tenen zwart worden, hetgeen kan leiden tot amputatie van de desbetreffende vinger of teen.

Op dit moment is er nog geen afdoende behandeling voor het fenomeen van Raynaud. Behandeling is gericht op het voorkómen van aanvallen door koude te vermijden en zich warm te kleden. Daarnaast worden vaatverwijdende medicijnen voorgeschreven met als doel verbetering van de bloeddoorstroming en hierdoor een afname in aantal en ernst van de Raynaud aanvallen. Veel patiënten hebben echter geen of onvoldoende baat bij de medicijnen, of staken ze in verband met bijwerkingen.
De oorzaak van het fenomeen van Raynaud is nog niet geheel opgehelderd. Meerdere factoren spelen een rol *(multifactorieel)*. Uit onderzoek is gebleken dat bij patiënten met het fenomeen van Raynaud, zowel de primaire als de secundaire vorm, verhoogde spiegels van een krachtige vaatvernauwer worden gevonden. Deze vaatvernauwer, endotheline-1 (ET-1), speelt ook mogelijk een rol bij de bindweefselvorming bij sclerodermie. Daarnaast is gebleken dat behandeling met een medicijn *(bosentan)* dat de vorming van het ET-1 remt , de vorming van nieuwe zweren aan de vingers voorkomt bij patiënten met sclerodermie. Ook zijn er mededelingen dat patiënten met het fenomeen van Raynaud baat hebben bij dit medicijn.

Hart- en vaatziekten
Bij sclerodermie zijn vaatafwijkingen aanwezig. Verschijnselen hiervan zijn het fenomeen van Raynaud, het ontstaan van digitale ulcera, een afname in aantal haarvaten *(capillairen)* in de nagelriem, en een afwijkende structuur van deze capillairen. De vaatafwijkingen zijn vooral aanwezig in de kleinere bloedvaten. Men vermoedt dat er door een virus of een chemische stof schade ontstaat aan de cellen *(endotheelcellen)* die de binnenkant van het bloedvat *(endotheel)* bekleden, of dat het eigen immuunsysteem geprikkeld *(geactiveerd)* raakt wat dan weer leidt tot schade aan de endotheelcellen. Endotheelcellen hebben een belangrijke rol bij het in stand houden van een normale vaatfunctie. Een slecht functionerend endotheel *(endotheeldysfunctie)* veroorzaakt verschuivingen in de balans die normaal gesproken aanwezig is, wat leidt tot o.a. samenklontering van bloedplaatjes *(plaatjesaggregatie)*, activatie van het stollingssyteem, vaatspasmen, en een verhoogde vaatdoorlaatbaarheid of lekkage *(permeabiliteit)* van de bloedvaten. Endotheelactivatie en endotheeldysfunctie zijn nog omkeerbare processen die uiteindelijk kunnen leiden tot structurele veranderingen in de bloedvaten. Endotheeldysfunctie speelt een belangrijke rol in het ontstaan van slagaderverkalking *(atherosclerose)*. Het is al langer bekend dat atherosclerose kan leiden tot hart- en vaatziekten. Naast bekende traditionele risicofactoren voor hart- en vaatziekten, zoals leeftijd, verhoogde bloeddruk *(hypertensie)*, roken, suikerziekte *(diabetes mellitus)*, verhoogde cholesterolwaarden *(hypercholesterolemie)* en overgewicht, is gebleken dat bij andere reumatische ziektebeelden, zoals reumatoide arthritis (RA) en systemische lupus erythematosus (SLE), ook niet-traditionele risicofactoren een rol spelen bij het ontstaan van atherosclerose. Bij niet-traditionele risicofactoren moet gedacht worden aan chronische ontsteking, gebruik van bepaalde medicijnen en aan andere, ziekte gerelateerde factoren. Uit onderzoek is ook gebleken dat stapeling van versuikerde eiwitten *(advanced glycation endproducts, AGEs)* in de huid een voorspellende waarde hebben voor het optreden van atherosclerose, toegenomen lekkage van de bloedvaten en zenuwstoornissen. Stapeling van AGEs kan ontstaan na perioden met verhoogde bloedsuikerwaarden, maar ook na perioden van ontsteking. Bij patiënten met RA en SLE is gevonden dat er een verhoogde stapeling van AGEs is in vergelijking met gezonde controles, waarbij er ook een relatie is met het optreden van atherosclerose.

In dit proefschrift is bij patiënten met sclerodermie onderzocht in welke mate endotheeldysfunctie aanwezig is, en in hoeverre traditionele risicofactoren voor hart-en vaatziekten hierin een rol spelen. Daarnaast is gekeken of blokkade van ET-1, een vaatvernauwend eiwit, een gunstig effect heeft op het aantal aanvallen en de ernst van het fenomeen van Raynaud en het dysfunctioneren van de kleine bloedvaten *(microcirculatie)*. Als laatste onderdeel is onderzocht of bij patiënten met sclerodermie atherosclerose meer voorkomt dan bij gezonde controles van dezelfde leeftijd, en of er een toegenomen stapeling is van AGEs.
In dit proefschrift worden verschillende vaat(functie)onderzoeken gebruikt om op deze vragen antwoord te krijgen.

Vaat(functie) onderzoeken
Een korte uitleg over de verschillende onderzoeken.
Laser Doppler fluxmetrie met iontoforese
Het vaatverwijdend vermogen van de kleine bloedvaatjes in de huid is bestudeerd met laser Doppler fluxmetrie (LDF) en iontoforese. LDF is een techniek waarbij met een ongevaarlijk laserlicht de bloedstroom in de bloedvaatjes die op ongeveer 1 mm van de huid liggen kan worden bestudeerd. Met een klein electrisch stroompje kunnen vaatstimulerende oplossingen door de huid toegediend worden *(iontoforese)*. Met LDF kunnen dan de vaatreacties gemeten worden. Er zijn twee verschillende vaatverwijdende stoffen gebruikt. De eerste stof, acetylcholine (ACh), geeft vaatverwijding doordat ACh het endotheel aanzet tot het produceren van vaatverwijders waardoor de onderliggende spierlaag van het bloedvat verslapt. Dit heet endotheel-afhankelijke vaatverwijding. De tweede stof, sodium nitroprusside (SNP) zorgt rechtstreeks voor spierverslapping, dus zonder de tussenkomst van het endotheel. Dit is de endotheel-onafhankelijke vaatverwijding.
Natrium fluoresceine videodensitometrie
De lekkage *(permeabiliteit)* van de capillairen wordt onderzocht door middel van natrium fluoresceine videodensitometrie. Via een infuus wordt een fluorescerende merkstof (natrium fluoresceine) toegediend. Tegelijkertijd wordt via een microscoop met een videocamera opnames gemaakt van de huid aan de binnenzijde van de enkel. Met behulp van de video-opnames kan bepaald worden wat de mate van lekkage is van de merkstof door de bloedvatwand. Daarnaast kan ook het aantal capillairen geteld worden.
Endotheelactivatie markers
In het bloed wordt gekeken naar markers voor activatie van de endotheelcellen. Deze markers zijn de von Willebrand factor (vWf), thrombomoduline (TM) en vascular cell adhesion molecule-1 (VCAM-1).
Nagelriem capillairoscopie
Met een microscoop wordt gekeken naar de capillairen in de nagelriem van de vingers. Met dit onderzoek kan het aantal capillairen en de vorm beoordeeld worden.
Photo-electro-plethysmografie
Bij dit onderzoek wordt de rechter hand eerst verwarmd tot 33°C en daarna in stappen van 3°C iedere 4 minuten afgekoeld tot 12°C . Daarna wordt de hand weer opgewarmd tot een temperatuur van 26°C gedurende een periode van 10 minuten. Met een apparaatje kan de bloeddoorstroming in de bloedvaten in de vingers tijdens de opwarming en afkoeling worden gemeten en geregistreerd.

Raynaud dagboek
In een dagboek worden door de patiënt dagelijks het aantal aanvallen, de duur van de aanvallen, en de ernst van de aanvallen (op een schaal van 0-10, waarbij 0 betekent dat er geen problemen in het dagelijks leven zijn door het fenomeen van Raynaud, en 10 dat er zeer ernstige problemen in het dagelijks leven zijn door het fenomeen van Raynaud) genoteerd.
IMT meting
Met echografie wordt de dikte van de binnenbekleding van de vaatwand (intima-*media dikte, IMT)* van de halsslagader *(arteria carotis)* gemeten. Hoe hoger de IMT waarde, hoe groter de kans op atherosclerose in het lichaam.
AGE-reader
De stapeling van AGEs kan gemeten worden met een apparaat, de zogenaamde AGE reader. Dit apparaat belicht de huid van de onderarm met ultraviolet licht. De huid heeft een natuurlijke fluorescentie *(autofluorescentie)*, en door het meten bij bepaalde lichtgolflengten kan de AGE-reader de autofluorescentie van de huid, welke goed overeenkomt met de stapeling van AGEs, bepalen.

Endotheeldysfunctie
Hoofdstuk 4 en **5** beschrijven de mate van endotheeldysfunctie bij patiënten met sclerodermie in vergelijking met gezonde personen. Hiertoe is gebruik gemaakt van LDF met iontoforese (**hoofdstuk4**) en de natrium fluoresceine videodensitometrie (**hoofdstuk 5**).
In **hoofstuk 4** worden de vaatreacties beschreven op ACh en SNP. De patiënten met sclerodermie hebben verlaagde vaatreacties op zowel ACh als SNP. De sclerodermie patiënten hadden echter ook vaker gerookt, gebruikten meer medicijnen voor een hoge bloeddruk en hadden verhoogde cholesterolwaarden. Dit zijn ook factoren die van invloed kunnen zijn op de vaatreacties in de microcirculatie. Ook waren de sclerodermie patiënten gemiddeld wat ouder. Door middel van een statistische analyse kan voor deze factoren gecorrigeerd worden en hieruit blijkt dat ook na correctie voor deze factoren de vaatreacties bij sclerodermie patiënten nog steeds verlaagd zijn. De verlaagde reacties op zowel ACh als SNP suggereren dat naast een abnormale functie van de endotheelcellen er ook een niet goed functionerende spierlaag is. Dit laatste zou veroorzaakt kunnen zijn door afwijkingen in de bloedvaten, bijvoorbeeld door vernauwingen of fibrosering.
In **hoofdstuk 5** wordt beschreven in hoeverre de toegenomen permeabiliteit van de capillairen, wat bij eerder onderzoek al is aangetoond in de nagelriemen, ook elders in het lichaam aanwezig is. Tot onze verrassing vonden wij echter bij sclerodermie patiënten een verminderde permeabiliteit, of lekkage, van de fluorescerende stof ter plaatse van de enkel in vergelijking met gezonde personen. De verminderde permeabiliteit blijkt met name verklaard te worden doordat er bij de patiënten met sclerodermie ook minder capillairen in de huid van de enkels aanwezig waren. Dat betekent dat afwijkingen aan de kleine bloedvaten ook elders in het lichaam voorkomen. We konden hierdoor echter niet bewijzen dat de permeabiliteit van een individuele capillair ter plaatse van de enkel is toegenomen.

Effect van bosentan op het fenomeen van Raynaud en endotheeldysfunctie
In **hoofdstuk 2** en **3** worden de resultaten beschreven van een onderzoek waarbij 15 patiënten met gelimiteerde cutane sclerodermie en het fenomeen van Raynaud gedurende 16 weken zijn behandeld met bosentan, een geneesmiddel dat de vorming van ET-1, een

krachtige vaatvernauwer, remt. Gegevens met betrekking tot de ernst van het fenomeen van Raynaud en de mate van endotheeldysfunctie werden bepaald vóór start van het onderzoek, 2 maal tijdens het onderzoek, en 4 weken na afloop van het onderzoek.
Hoofdstuk 2 beschrijft het effect van het gebruik van bosentan op het fenomeen van Raynaud. Patiënten hadden duidelijk minder aanvallen, die minder lang duurden en ook als minder ernstig werden ervaren. Het is bekend dat patiënten met het fenomeen van Raynaud minder klachten hebben als de buitentemperatuur hoger is. Alhoewel de buitentemperatuur aan het einde van de studie duidelijk hoger was dan aan het begin van de studie lijkt het niet waarschijnlijk dat dit een effect had op de resultaten aangezien de verbetering al eerder aanwezig was. Er werd echter geen verbetering gevonden van de doorbloeding van de vingers bij het afkoelingsonderzoek. Ook in andere studies waar vaatverwijders werden onderzocht, kon de verbetering in het fenomeen van Raynaud niet altijd geobjectiveerd worden. Omdat er in deze studie geen controlegroep aanwezig was die geen placebo medicijn kreeg, kan een placebo-effect niet worden uitgesloten
Wij konden, zoals beschreven in **hoofdstuk 3**, ook geen effect waarnemen op de endotheeldysfunctie. Er waren geen verbeteringen in het vaatverwijdend vermogen van de microcirculatie, de permeabiliteit, en het aantal capillairen in de nagelriem. Ook waren de markers voor endotheelactivatie niet duidelijk veranderd, met uitzondering van één van de markers. Het is mogelijk dat de duur van de studie niet lang genoeg is geweest om het effect op het endotheel waar te nemen.

Vroegtijdige atherosclerose
In **hoofdstuk 6** geven we een overzicht van de onderzoeken die het vóórkomen van (vroegtijdige) atherosclerose bij patiënten met sclerodermie beschrijven. Er worden meer afwijkingen aan de slagaders van de vingers en onderarm gevonden bij patiënten met sclerodermie, maar er is geen duidelijke toename in het vóórkomen van hart- en vaatziekten. Wanneer de IMT waarde gebruikt wordt als maat voor atherosclerose en de kans op hart- en vaatziekten, dan worden er wisselende resultaten gevonden. Sommige onderzoekers vinden verhoogde IMT waarden, terwijl andere onderzoekers geen verschil vinden met IMT waarden gemeten bij gezonde controles. Dit is in tegenstelling tot andere reumatische aandoeningen zoals reumatoide arthritis en SLE. Bij deze ziekten wordt een verhoogd risico op atherosclerose met een verhoogde kans op hart- en vaatziekten gevonden. Naast de bekende traditionele risicofactoren spelen ziekte-gerelateerde factoren hierin ook een belangrijke rol. Deze ziekte-gerelateerde factoren zijn ook bij sclerodermie aanwezig, dus een duidelijke verklaring voor het gevonden verschil met andere reumatische aandoeningen is er op het moment nog niet.
In **hoofdstuk 7** is gekeken of er vroegtijdige atherosclerose is bij sclerodermie patiënten ten opzichte van gezonde personen. Een IMT meting werd verricht en zowel traditionele risicofactoren voor hart- en vaatziekten als ziekte-gerelateerde factoren werden bepaald. Ook in ons onderzoek werd geen verschil gevonden tussen IMT waarden bij sclerodermie patiënten en gezonde controles. De groepen verschilden wat betreft traditionele risicofactoren, bijvoorbeeld een hogere leeftijd en een meer frequent gebruik van cholesterolverlagende medicijnen bij sclerodermie patiënten. Hiervoor werd gecorrigeerd in de statistische analyse, maar ook dan kon er geen verschil in IMT waarden tussen sclerodermie patiënten en gezonde controle personen worden aangetoond. Alhoewel er veel vaatproblemen voorkomen bij patiënten met sclerodermie lijken die met name zich te

bevinden in de kleine bloedvaatjes en niet in de grotere bloedvaten. Een toegenomen risico op atherosclerose kon niet worden aangetoond.

Advanced Glycation endproducts

In **hoofdstuk 8** worden de resultaten beschreven betreffende de versuikerde eiwitten (*AGEs*) bij sclerodermie patiënten en controles. Zoals er eerder geen hogere IMT waarden werden gevonden bij sclerodermie patiënten in vergelijking met controles, kon er nu ook geen verhoogde stapeling van AGEs worden aangetoond. Wel was er, net zoals in eerder onderzoek, een relatie tussen de mate van stapeling van AGEs en de leeftijd. Bij sclerodermie patiënten was er mogelijk ook een verband met de mate van ziekte-ernst, ziekte-activiteit en schade ten gevolge van de ziekte, de hoeveelheid van één van de ontstekingseiwitten in het bloed (*C-reactief protein, CRP*), en het gebruik van bepaalde bloeddrukverlagende medicijnen (zogenaamde ACE remmers en AII antagonisten). Behalve de bloeddrukverlagende medicijnen en de al eerder genoemde leeftijd bleken de overige ziekte-gerelateerde factoren bij verdere statistische analyse niet van belang te zijn voor de mate van stapeling van AGEs. Het is niet bekend in hoeverre de huidveranderingen bij sclerodermie zelf van invloed zijn geweest op de meetuitslagen. De meting van de autofluorescentie van de huid is een betrouwbare maat voor stapeling van AGEs bij gezonde personen en bij personen met verschillende ziektebeelden, waaronder diabetes mellitus en nierziekten. Het is mogelijk dat de fibrosering van de scleroderme huid, alhoewel niet duidelijk aanwezig op de plaats van de meting, de meting heeft beïnvloed. Ook kan het gebruik van ACE remmers en AII antagonisten geleid hebben tot lagere waarden, omdat uit onderzoek is gebleken dat het gebruik van deze medicijnen kan leiden tot lagere waarden van AGE stapeling. Bepaling van de stapeling van AGEs lijkt op dit moment nog geen toegevoegde waarde te hebben bij het onderzoek naar endotheeldysfunctie en atherosclerose bij sclerodermie patiënten.

Conclusie en toekomstperspectief

Sclerodermie is met name een ziekte van de kleine bloedvaten gezien de aanwezigheid van het fenomeen van Raynaud, de afwijkingen aan de capillairen van de nagelriem, het afgenomen aantal capillairen in de nagelriem, en de afwijkingen van de endotheelfunctie in de microcirculatie. Afwijkingen in het aantal capillairen en de permeabiliteit werden ook gevonden elders in het lichaam, namelijk ter plaatse van de enkel. De vraag is dan ook of dit geldt voor alle kleine bloedvaten in het gehele lichaam. Bekende risicofactoren voor hart-en vaatziekten (zoals bijvoorbeeld diabetes mellitus, hypercholesterolemie) lijken geen belangrijke rol te spelen bij het ontstaan van afwijkingen aan de kleine bloedvaten. Toch lijkt het wel zinvol en nuttig om deze risicofactoren goed te behandelen. Ander onderzoek toonde aan dat cholesterolverlagende medicijnen *(statines)* mogelijk een gunstig effect hebben op het voorkómen van endotheeldysfunctie. Verder onderzoek is nodig om te beoordelen of het gebruik van statines of andere medicijnen vroeg in de ziekte een gunstig effect heeft op het verminderen van problemen in de kleine bloedvaten en zorgt voor een verbetering van de kwaliteit van leven.

Het fenomeen van Raynaud bij sclerodermie patiënten reageerde gunstig op het gebruik van bosentan, maar het betrof een studie waarbij er geen controlegroep aanwezig was. We konden het gunstige effect op het fenomeen van Raynaud niet objectiveren door middel van het verrichtte vaatonderzoek. In andere studies, waarbij wel een controle groep aanwezig

was, werd overigens gevonden dat bosentan geen effect had op het fenomeen van Raynaud en soms ook niet op de endotheel dysfunctie. Wij konden ook geen effect aantonen van het medicijn op de functie van het endotheel. Wellicht is de duur van de behandeling te kort geweest om het effect te kunnen waarnemen, of kan het ontbreken van een effect te maken hebben met de aanwezigheid van reeds irreversibele schade aan de bloedvaten van de door ons onderzochte groep sclerodermie patiënten. Een langer durende studie waarin ook een controlegroep aanwezig is die geen bosentan gebruikt is nodig om aan te tonen of er een effect is op het fenomeen van Raynaud en de endotheelfunctie van de microcirculatie. Overigens dient een dergelijk onderzoek bij voorkeur plaats te vinden bij sclerodermie patiënten die een korte ziekteduur hebben om de kans op structurele vaatschade in de te onderzoeken groep te verkleinen.
In tegenstelling tot andere reumatische ziekten, zoals bijv reumatoide arthritis en SLE, is er geen toegenomen risico op vroegtijdige atherosclerose. IMT, als maat voor vroegtijdige atherosclerose, is bij sclerodermie patiënten niet verhoogd in vergelijking met een gezonde controle groep. Waarom het risico op atherosclerose en afwijkingen aan de grotere bloedvaten bij sclerodermie niet evident is toegenomen is niet bekend. Mogelijk speelt de mate van ontsteking in het lichaam hierin een rol, die bij sclerodermie minder op de voorgrond staat dan bij de andere reumatische ziekten. Desondanks moeten traditionele risicofactoren voor hart- en vaatziekten wel actief opgespoord en behandeld worden aangezien het bekend is dat deze invloed hebben op endotheeldysfunctie en het optreden van atherosclerose.
Het meten van stapeling van AGEs is bij patiënten met sclerodermie op dit moment nog niet van toegevoegde waarde. Onderzoek zal nog moeten volgen om te beoordelen in hoeverre de meting betrouwbaar is bij patiënten met sclerodermie. Een relatie tussen leeftijd en mate van stapeling van AGEs was echter wel aanwezig. Voor andere patiëntengroepen binnen de reumatologie lijkt het bepalen van AGEs een eenvoudige manier om een indruk te krijgen van vroege atherosclerose.

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Dankwoord

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