THESIS FOR THE DEGREE OF DOCTOR OF PHILOSOPHY (PhD)

Assessment of cardiovascular abnormalities and the effect of rosuvastatin treatment in patients with systemic sclerosis

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ABBREVIATIONS

5-HT	5-hydroxy triptamine, serotonine
α-AR	alpha adrenergic receptor
α-SMA	alpha-smooth muscle actin
AB(P)I	ankle-brachial (pressure) index
ACC	American College of Cardiology
ACE	angiotensin converting enzyme
ACR	American College of Rheumatology
ADAMTS-13	A Disintegrin And Metalloproteinase with a Thrombospondin type 1 motif, member 13, or von Willebrand factor-cleaving protease
ADMA	asymmetric dimethylarginine
AECA	anti-endothelial cell antibodies
AHA	American Heart Association
AI	atherogenic index (TG/HDL)
AIx	augmentation index
ANA	anti-nuclear antibodies
ARB	angiotensin receptor blockers
ASTEROID	A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden
ATA	anterior tibial artery
ATS	American Thoracic Society
AT-II	angiotensin II
ATP	posterior tibial artery
BAD	brachial artery diameter
BMI	body mass index : weight in kilograms divided by (height in meters)^2
BNP	B-type natriuretic peptide
C3, C4	complement-3 factor and 4 factor
CAD	coronary arterial disease
CAM	cellular adhesion molecule
CC	covariation coefficient
ccIMT	intima-media thickness of the common carotid artery
CGRP	calcitonin gene related peptide
CRP	high sensitivity C-reactive protein
CTD	connective tissue disease
CV	cardiovascular
CW Doppler	continuous wave Doppler
CXCL4 CXC	chemokine ligand 4
DC	diffusion capacity
dcSSc	diffuse cutaneous systemic sclerosis
DDAH2	dimethylarginine dimethylaminohydrolase 2 gene, the encoded protein of which plays a role in regulating cellular concentrations of methylarginines, inhibitors of NO synthase activity
DLCO	diffusion lung capacity for carbon monoxide
DT	deceleration time
ECG	electrocardiogram
EF	ejection fraction
ELISA	enzyme-linked immunosorbent assay
ENA	extractable nuclear antigen
eNOS	endothelial NO synthase
EPC	endothelial progenitor cell
ESR	erythrocyte sedimentation rate
ET-1	endothelin-1
EULAR	European League Against Rheumatism
FGF	fibroblast growth factor
FLI1	Friend leukemia virus integration-1
FMD	flow-mediated vasodilation
FRA2	Fos-related antigen 2
GFR	glomerular filtration rate
GTP	guanosyne triphosphaete
HAQ	health assessment questionnaire

HDL	high density lipoprotein
HIF-1	hypoxia-inducible factor-1
HLA	human leukocyte antigen
HMGB-1	High-mobility Group Box 1, a nuclear protein regulator of cell death and survival
HMG-coA	3hydroxi-3methyl glutaryl- coenzyme-A
HMWMAA	high molecular weight melanoma-associated antigen
HR-CT	high resolution computed tomography
HRV	heart rate variability
I(V)CT	isovolumic contraction time
I(V)RT	isovolumic relaxation time
IC	immune complex
ICC	intraclass coefficient
IFN-α, IFN-γ	interferon alpha, interferon gamma
IL-4, IL-6	interleukin-4, interleukin-6
ILD	interstitial lung disease
iPAH	idiopathic pulmonary arterial hypertension
IVUS	intravascular ultrasound
Jo-1	antibody against histidyl-tRNA synthetase
LAH	left anterior hemiblock
LBBB	left bundle branch block
lcSSc	limited cutaneous systemic sclerosis
LDL	low density lipoprotein
LDPM	laser Doppler perfusion monitoring
LV	left ventricular
LVH	left ventricular hypertrophy
MDRD	Modification of Diet in Renal Disease study
MHC	major histocompatibility complex
MMP-12	matrix metalloproteinase-12
MPI	myocardial performance index, TEI index
mRSS	modified Rodnan skin score
MTFR	methylene tetrahydrofolate reductase
NADPH	nicotinamide adenine dinucleotide phosphate
NMD	nitrate-mediated vasodilation
NO	nitric oxide
NSAID	nonsteroid anti-inflammatory drugs
NT- pro-BNP	N-terminal of B-type natriuretic peptide (BNP)
OR	odds ratio
PAD	peripheral arterial disease
PAH	pulmonary arterial hypertension
PAI-1	plasminogen activator inhibitor-1 or serine protease inhibitor-E1
PAP	pulmonay arterial pressure17
PDE-5	phosphodiesterase-5
PDE5	phosphodiesterase-5
PDGFR-β	platelet derived growth factor receptor beta
PGE-5	prostaglandine E 5
PGI2	prostacyclin
PlGF	placental growth factor
PORH	post-occlusive reactive hyperemia
PP	pulse pressure
PPAR-γ	peroxisome proliferator-activated receptor gamma
PU	perfusion unit
PW Doppler	pulsed wave Doppler
PWV	pulse wave velocity
QTc	QT time, corrected for 60/min heart rate
RA	rheumatoid arthritis
RBBB	right bundle branch block
RGS5	regulator of G-protein signaling-5
RNP	ribonucleoprotein
ROCK	Rho-associated protein kinase
ROS	reactive oxygen species
RP	Raynaud’s phenomenon

RV	right ventricular
Scl-70	anti–topoisomerase antibodies
SCORE chart	Systematic COronary Risk Evaluation (a European risk chart based on gender, age, total cholesterol, systolic blood pressure and smoking status)
S.D.	standard deviation, amount of variation or dispersion from the average
SLE	systemic lupus erythematosus
Sm	Smith antigen
SMC	smooth muscle cell
SPARC	secreted protein, acidic, cysteine-rich
SPSS	statistical package for the social sciences
SS-A	Sjögren`s syndrome-A antigen/ Anti-Ro, an antinuclear antibody target
SS-B	Sjögren syndrome`s B antigen / anti-La, an antinuclear antibody target
SSc	systemic sclerosis
SVES	supraventricular extrasystole
TAPSE	tricuspid annular plane systolic anterior excursion
TG	triglyceride
TGF-β	transforming growth factor beta
TH1	time to half before hyperemia
TH2	time to half after hyperemia
TM	time to maximum flow from deflation during laser Doppler PORH testing
TNF	tumor necrosis factor
TXA2	thromboxane A2
u-PAR	urokinase-type plasminogen activator receptor
VC	variation coeffitient
VCAM	vascular cell adhesion molecule
VE-cadherin	vascular endothelial cadherin
VEGF	vascular endothelial growth factor
VES	ventrcicular etrasystole
VT	ventricular tachycardia
vWF-Ag	von Willebrand factor antigen

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1. INTRODUCTION

1.1. Definition of SSc

Systemic sclerosis (SSc, scleroderma) is a chronic connective tissue disease (CTD) characterized by autoimmune features, functional microvascular impairment and fibrosis of the skin and several internal organs, such as lungs, kidneys, gastrointestinal tract and the heart. The first, inexact description of the symptoms was given by Hippocrates as early as 400 B.C., although the next written reference of the illness, a case report of a 17-year old female patient was published much later, in 1753, by Carlo Curzio in Italy. The systemic characteristic of the disease was recognized by Sir William Osler towards the end of the 19th century but the term ’systemic sclerosis’ was introduced by Goetz as late as 1945. For decades, classification criteria applied to identify SSc patients were those established in 1980 by the American College of Rheumatology (ACR) [1]. In 2013, classification criteria of SSc were rewised by members of the ACR and EULAR (ACR/EULAR 2013 criteria, [2]), new criteria were introduced, thus, sensitivity and specificity of the diagnosis improved to 90%. In addition to proximal scleroderma, sclerodactily, fingertip ulcers or digital scarring and bibasilar pulmonary fibrosis used by the former criteria, since 2013, teleangiectasies, abnormal nailfold capillaries, puffy fingers, pulmonary arterial hypertension, Raynaud-syndrome and certain autoantibodies (anti-centromere, Scl-70, anti-RNA polymerase-3) are taken into accoung to establish the diagnosis of SSc. Since all patients participating in the studies discussed within these thesis were diagnosed with SSc prior to 2013, we will refer to the 1980 ACR diagnostic criteria in the Patients and methods section.

1.2. Epidemiology

The worldwide prevalence of the disease varies between 19-472/100,000, latter in Choctaw Native Americans, depending on geographical location, race, gender, age of the studied population [3]. The current prevalence of SSc in Hungary, as reported by Czirják et al [4], is around 91/10,000. This group screened 10,000 Hungarian adults by face-to-face interviews for Raynaud’s phenomenon (RP) and performed capillaroscopy in patients complaining about clinically significant RP, performing immunolaboratory assessment if necessary. The 9 ‰ prevalence of SSc is significantly higher than previously presumed.

Despite earlier diagnosis, increasing knowledge about pathogenesis, and some emerging novel treatment options brought by the past decades, SSc remains one of the most devastating among CTDs. Considerable mortality rate of the disease is represented by a pooled standardized mortality ratio of 3.5 [5], which has improved slowly during the past 40 years. During this period, the 10-year survival of SSc patients improved first from 54% to 66% and lately, according to newly published italian population based studies, further improvement in 10-year survival to as much as 83.5% for patients diagnosed after 1999 has been reported [6]. Non-SSc related causes of death became more frequent, accounting nowadays for almost a half of all cases, as opposed to 20% in the 1970s. Today, the survival of SSc patients is mainly determined by internal organ involvement. The most common SSc-related causes of death include cardiac (20%, mostly heart failure and arrhythmias), interstitial lung disease (ILD; 17%), pulmonary arterial hypertension (PAH; 13%) and renal disease (14%) [7] (**Figure 1)**.

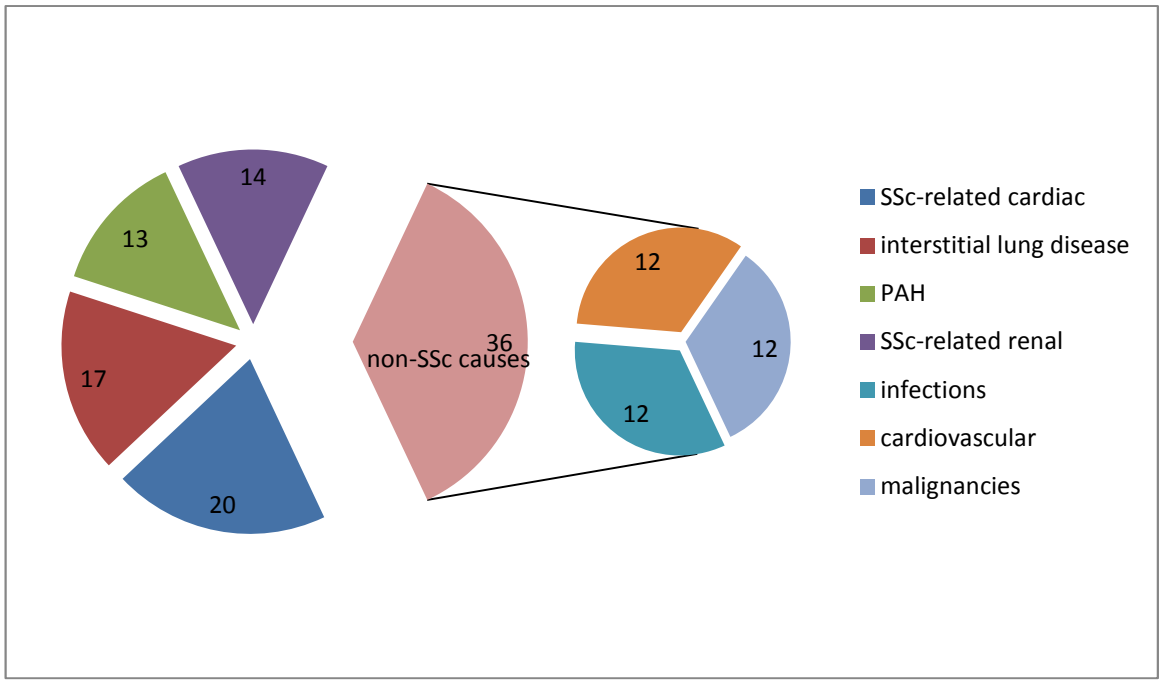

Figure 1. Causes of death in SSc (2012). SSc-related cardiac involvement refers to diagnosed cardiac disease (myocardiac fibrosis, decreased coronary reserve capacity, diastolic or systolic dysfunction, conduction disturbances) in absence of an evident underlying (non-SSc related) cause explaining cardiac disease. Such causes could be interstitial lung disease, pulmonary arterial hypertension, systemic hypertension, or severe renal disease, but also epicardiac coronary atherosclerosis, which can occur just as frequently as in the general population. Cardiac involvement in SSc without any of the above diseases is postulated to be a consequence of microvascular involvement and ischemia, hence is attributed to SSc itself.[10].

The introduction of ACE-inhibitors diminished occurrence of scleroderma renal crisis dramatically, resulting in a drop in renal causes of death from 42% to 6% by the year 2002. [8] The treatment of PAH patients with potent modern vasodilators, such as endothelin 1 (ET-1) receptor

antagonists, prostacyclin analogues and inhibitors of phosphodiesterase-5 (PDE5) improved two-year survival substantially compared to survival obtained with earlier treatments. Yet, the 3-year survival still remains poor [9]. Remarkably, currently 36-41% of mortality is caused by non-SSc-related diseases, approximately evenly distributed between cardiovascular (CV) causes, malignancies and concomitant infections. This indicates an increasing significance of cardiac and vascular involvement, excluding PAH, adding up to account for one-third of total mortality, thus conveying the greatest risk in the following years for SSc patients among all causes [7].

Tyndall et al [10] reported similar results from EULAR centers participating in a study covering 5860 patients with SSc in 2010. Altogether 55% of mortality belonged to SSc-related, 41% to non-SSc causes, while in 4%, the cause of death could not be defined. In the aforementioned report, most frequent SSc-associated deaths occurred due to pulmonary fibrosis (20% of overall mortality), PAH (14.3%) and cardiac failure or arrhythmias (26%).

Although internal organ involvement, microvascular and pulmonary arterial abnormalities have been widely investigated in SSc, until the past two decades, cardiac and macrovascular involvement remained poorly studied in this disease.

After I have graduated as M.D. in Budapest in 2004, I moved to Debrecen to start working at the Third Department of Internal Medicine of the University. My attention turned early to non-invasive CV imaging. Both the less broadly studied macrovascular alterations, as well as significant mortality in SSc contributed to the fact, that in the past years, as a result of a fruitful cooperation between the Department of Angiology and the Department of Rheumatology at the University of Debrecen, I took the opportunity to search for less evident signs of macrovascular and cardiac dysfunction in a group of Hungarian patients with SSc. We also investigated the clinical effects of rosuvastatin therapy on this particular patient cohort. Before performing original studies, I have reviewed the current literature in this research area.

2. LITERATURE REVIEW

2.1. Pathophysiology of vascular disease in SSc

Despite extensive research in the field of rheumatology and well-coordinated clinical management of SSc, the exact pathomechanism of this disease is still unclear. In the following sections, I will review current knowledge on vascular dysfunction and triggers of CV disease in SSc.

2.1.1. Genetic predisposition and environmental factors

Although mild familial aggregation of SSc have been described suggesting a weak genetic predisposition to the disorder, identical twin studies show a low concordance [11]. First-degree relatives of SSc patients have an increased risk (3-5%) of developing autoimmune thyroid disease, rheumatoid arthritis (RA), diabetes or other CTDs, such as SLE, SSc and others [12], suggesting a common pool of genetic disorders rather than a specific mutation in the background of the disease. Candidate loci responsible for genetic predisposition include the HLA region (DR52 can be linked to pulmonary fibrosis), chromosome 5 (*SPARC* gene), chromosome 15 (fibrillin gene defect) or cytokine receptor regions, as well as genes connected to collagen synthesis.

On the other hand, it has been noted, that spouses of SSc patients display an increased prevalence of the disease as well, indicating additional role of specific environmental factors[13]. Environmental background of such rare diseases is very difficult to study, thus data or large-scale studies on this topic are scarce. The most important environmental hazards identified in the history of Hungarian SSc patients included exposure to organic solvents. In addition, silica exposure, epoxy resins, aromatic carbonhydrogenes, welding fumes, specific drugs (pentazocine, bleomycine) or physical hazards such as vibration are also possible triggers of SSc. Association of SSc with presence of infectious agents or maternal microchimerism has also been suggested .

2.1.2. Vascular dysfunction

Functional abnormalities, such as disequilibrium of vascular tone, overexpression of cellular adhesion molecules (CAMs) and structural vascular pathology, including defective angiogenesis, pathological electronmicroscopic and histological structure, in particular endothelial cell, pericyte and basement membrane disorders, are the hallmark of the disease and appear in the initial phase of disease pathogenesis. [14]

2.1.2.1. Endothelial dysfunction

Endothelial dysfunction seems to be among the first alterations observable in SSc. The vascular endothelium is an important regulator of vessel wall homeostasis, maintaining relaxation of smooth muscle tone and limiting oxidative stress via nitric oxide (NO), prostacyclin (PGI2), ET-1 release and influencing vascular angiotensin II (AT-II) activity. One source of NO is from conversion of L-arginine by the endothelial NO synthase (eNOS), another source is the inducible NO synthase of macrophages, which can also produce NO in case of various immunological impulses. In addition, the endothelium plays an important role in the maintenance and control of vascular permeability to plasma components and platelets, as well as white blood cell adhesion, aggregation and thrombosis [15]. “Endothelial activation” and “endothelial dysfunction” describe changes in normal homeostatic control of the endothelium in response to harmful stimuli, resulting in phenotypic changes, such as expression of various cellular adhesion molecules, inflammatory cytokine production, vasoconstriction, increased oxidative stress and produce of pro-thrombotic substances. These pathophysiologic changes are detectable via functional imaging techniques such as impaired flow-mediated vasodilation (FMD) of the brachial artery in the clinical setting.

2.1.2.2. Impaired vascular tone

In SSc, an imbalance between physiologic vasoconstrictors and vasodilators setting the vascular tone evolves resulting in a shift in favour of vasoconstriction. ET-1, a potent vasoconstrictor is increased in SSc while levels of the vasodilator NO and PGI2 are decreased in the disease [16]. Neural abnormalities, such as increased α adrenergic receptor (α-AR) activation have also been suggested to contribute to impaired vasodilation [17]. Clinical significance of α-AR activation in SSc resides in that sympathetic tone is the most important neurogenic regulator of peripheral vascular tone. It has been demonstrated that α2-type adrenoreceptors are overexpressed in SSc patient’s cutaneous circulation. The α-2c AR-subtype is translocated in response to cold exposure from the Golgi apparate to the cell membrane and undergoes rapid selective activation. Cold stimuli lead to smooth muscle cell (SMC) mitochondrial reactive oxygen species (ROS) production and Rho/Rac kinase activation (ROCK pathway) which in turn takes part in α-2c AR translocation, but also fibroblast differentiation and extracellular matrix (ECM) production, which is why it has been suggested as a therapeutic target in SSc [18, 19].

On the other hand, overexpression of CAMs, such as intercellular cell adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1) and E-selectin by endothelial cells, as well as elevated production of soluble CAMs have been detected in patients with SSc. CAMs pave the

way for increased capillary permeability (edema), thus platelet and inflammatory cell transmigration (activated T-lymphocytes, macrophages, basophil lymphocytes) to the vessel wall [16, 17].

2.1.2.3. Defective angiogenesis

Impaired angiogenesis has been described in SSc despite constant presence of increased proangiogenic factors, most importantly vascular endothelial growth factor (VEGF), but also platelet-derived (PDGF), placental (PlGF) and fibroblast growth factors (FGF-2), as well as ET-1, in the sera of SSc patients. Increased VEGF expression seems to be independent from tissue hypoxia, according to a study reporting about reduced hypoxia-inducible factor-1 (HIF-1) levels in SSc-affected skin biopsies [20]. Elevated VEGF levels have been found to correlate with severity of organ involvement, thus represent possible prognostic information in SSc [21]. Along with pro-angiogenic factors, in certain disease subsets, enhanced release of angiostatic molecules, such as angiostatin, CXC chemokine ligand 4 (CXCL4), thrombospondin and IL-4, have also been described, but the pathological imbalance between angiogenic and angiostatic mediators favour impaired neovascularization leading to skin ulcers and other pathologies. Dermal endothelial cells of SSc patients were reported to show abundant interferon α (IFN-α) signaling, possibly contributing to angiostatic and perhaps pro-apoptotic effects in the SSc-affected endothelium [22]. Studies of endothelial cells from SSc-affected dermis conveyed two additional results of importance. The first is that vascular endothelial (VE-) cadherin, an important constituent of tight junctions, is absent in SSc dermal specimen, resulting in a deficient barrier function of the affected endothelium and subsequent edema formation [22]. In addition, the urokinase type plasminogen activator receptor (uPAR) seems to explain why despite a pro-angiogenic gene expression pattern, impaired endothelial-cell mediated angiogenesis has been associated with SSc [23, 24]. Moreover, SSc fibroblasts inhibit normal vasculogenesis. Low numbers of circulating endothelial progenitor cells (EPC) have also been detected accounting for ineffective vasculogenesis in SSc [17, 25]

2.1.2.4. Cellular and structural abnormalities

Platelet activation is linked strongly to fibrosis and immune activation in SSc [26]. Moreover, recent research has identified possible therapeutic targets that might interfere with fibrogenesis in SSc. Platelet activation following vascular dysfunction leads to production of thromboxane A2 (TXA2) and other vasoactive mediators contributing to vasoconstriction and thrombosis. Fibrotic tissue further enhances platelet activation via type I collagen, wheras platelets promote fibrosis via serotonine (5-HT), PDGF, transforming growth factor β (TGF-β) and lisophospholipid production. 5-

HT is a molecule of outstanding importance in SSc, since it has recently been reported to activate fibroblast collagen production via the receptor $5HT_{2B}$. In mouse models, inhibition of this receptor blocked fibrosis, indicating clinical relevance of the above pathway in disease pathogenesis and therapy [27, 28]. Clinical trials with 5HT-antagonists are also being conducted . Circulating platelet-leukocyte heterotypic aggregates play a role in inflammation and autoimmune processes of pathogenesis [29-31], while IFN-γ released by the autoreactive T-cells are hypothesized to change megakaryocyte maturation and basal platelet activity .

Structural analysis of the basement membrane of SSc-affected skin specimens have shown decreased levels of type IV collagen compared to healthy skin. [26, 32]. In addition to endothelial cells and fibroblasts, pericytes have also been indicated among cellular components involved in early SSc pathogenesis. SSc-related, activated pericytes express immature pericyte markers PDGF receptor β (PDGFR-β), high molecular weight melanoma-associated antigen (HMW-MAA), as well as regulator of G-protein signaling-5 (RGS5), which may be a negative regulator of vessel maturation. Levels of α-smooth muscle actin (α-SMA), characteristic of mature pericytes and mature fibroblasts, in contrary, are reduced in capillaries and venules of SSc patients [33]. As discussed later, the above mentioned mechanisms yield to various morphologic vascular malformations, such as teleangiectasies, a reduction in capillary numbers, avascular areas, all associated with SSc.

The invasion of ECM by inflammatory cells leads to the production of various cytokines including members of the TGF-β family, PDGF, interleukin 1 (IL-1), IL-4, IL-13, tumor necrosis factor α (TNF-α), as well as that of other mediators, such as ET-1 or the adipokine leptin. These mediators amplify the inflammatory response and also recruit and activate fibroblasts in the SSc-affected tissue eventually leading to obstructive fibrotic vascular lesions and further tissue ischemia . As also discussed later, the precise link between vascular injury and tissue fibrosis in SSc is still debated. According to a current hypothesis proposed by Trojanowska et al [34, 35], vascular injury caused by various triggers leads to an increased vasoconstriction and expression of CAMs on the surface of endothelial cells. This renders immune-inflammatory cell transmigration possible and leads to production of pro-angiogenic molecules, of which VEGF is of outstanding importance. However, due to certain specific endothelial cell disorders in SSc, such as absence of VE-cadherin and cleavage of uPAR leading to aberrant cell-cell junctions and pathologic angiogenesis, pro-angiogenic mediators are unable to promote physiologic angiogenesis in SSc. Activated endothelial cells, epithelial cells and pericytes might trans-differentiate into fibroblasts in SSc, which cells, along with resident fibroblasts and fibrocytes recruited from the circulation or bone marrow lead to increased collagen production and tissue fibrosis [15].

2.1.3. Atherosclerosis and generalized vasculopathy in SSc

Vasculopathy characteristic of SSc appears to be distinct both in histopathological presentation, localisation and in cellular-molecular background from the accelerated atherosclerosis observable in SLE or RA. Subintimal proliferation and fibrosis, along with preserved media and leukocyte infiltration of the vessel wall characterizes pathologic SSc vessels. Limited cutaneous SSc (lcSSc) is thought to be more prone to peripheral vascular involvement, while the diffuse form (dcSSc) of the disease is characterized by increased prevalence of early, severe internal organ involvement and scleroderma renal crisis. Despite overt signs of SSc-vasculopathy, in the past few years, an ongoing debate exists wether or not signs of coronary, carotid or peripheral atherosclerosis, or increased frequency of surrogate markers of atherosclerosis, e.g. endothelial dysfunction and abnormal arterial stiffness can be observed in SSc [36, 37]. In general, it has been accepted that early and generalized atherosclerosis has not been consistently reported in SSc. Early signs of atherosclerosis such as endothelial dysfunction, arterial stiffness and increased ccIMT will be discussed later in relation to SSc. Hereby we will give an overview of data about coronary atherosclerosis in SSc.

2.1.3.1. Coronary artery disease (CAD) and microvasculopathy of the heart

The exact prevalence and incidence of CAD in SSc is currently unkown. Silent or functional cardiac disorders, as well as atypical presentation of symptoms and overlap between diseases such as between CAD and PAH [38, 39] account for uncertainty. **Table 1.** summarizes results of four studies addressing CAD prevalence among SSc patients.

Table 1. Coronary artery disease prevalence in SSc

Study(Author, Ref., Year)	Number of Patients	CAD prevalence (%)	Other findings
Komocsi et al, 2010	120	15/120 (12.5%)	Of 120 patients, 30 underwent coronary angiography after screening. PAH prevalence was 14/120 (11.6%)
Bulkley et al,[39, 40] 1976	52	4% had extramural coronary arterial disease	Of 52 patients, 26 (50%) had myocardial necrosis foci on autopsy with open extramural coronary arteries (SSc-microvascular involvement)
Akram et al,[41] 2006	172	22%	172 coronary angiographies were performed due to dyspnoae (67%), typical (16%) or atypical (17%) angina; 22% indicated significant stenosis
Tarek et al, [42] 2006	14	21.4%	3 out of 14 asymptomatic, female SSc patients had significant coronary stenosis on coronary angiography

Briefly, CAD prevalence among SSc patients who underwent coronary angiography was 18.3% (significant stenosis) as opposed to the 4% rate from autopsies. The discrepancy is probably due to better treatment of SSc-specific symptoms in the past few years, thus more time for coronary involvement to develop. Although the rate of CAD is increasing in scleroderma, it appears, that accelerated coronary atherosclerosis as observable in RA or SLE is not typical for SSc [37, 43].

In 2013, Zubieta et al presented interesting data after comparative retrospective analysis of 1208 individuals with SSc and 12 080 age, sex, and follow-up year matched controls out of 5 million patients' data belonging to the same geographical province [44]. Among the selected SSc patients, 90 developed acute myocardial infarction, resulting in an incidence of 20.2 per 1000 patient years. Compared to non-SSc individuals, the overall relative risk for MI was 3.8, and remained significant (4.0) even if adjustment for comorbidities was performed. A key finding, according to the presentation is that relative risk was detected to be 9-fold within the first year of SSc diagnosis, 3.0 between a disease duration of 1-5 years and further decreases to 1.6 over time in patients grater than 5 years of disease duration. The greatest incidence rate was detected in the 45-59 age group (5.2).

The explanation of increased MI rates inspite of normal coronary angiograms typical in SSc might be related to the decreased coronary reserve capacity of SSc patients. We can hypothethise that since SSc patients' myocardial blood supply is compromised, it is more sensitive to even transient ischaemia thus a definitive MI is more likely to develop compared to controls.

2.1.3.2. Peripherial arterial disease (PAD)

Following occasional reports of upper or lower limb peripheral obstructive arteriopathy (ulnar artery, popliteal artery) [45, 46], more extensive reports in search of general macrovascular disease in SSc have been published [47-49]. Although some results indicated increased PAD prevalence in SSc vs. controls (58% vs. 9.8%) [48], others found solely ulnar arteriopathy [49] upon neck, upper and lower limb arterial Doppler ultrasound assessments. The distribution of atherosclerotic or stenotic arteries is uneven in SSc patients. Arteriopathy or arteriosclerosis of the great elastic arteries, the aorta and its main branches (brachiocephalic artery, left common carotid and subclavian artery) as well as the main pulmonal artery is unusual in SSc [42, 43].

2.2. Clinical presentation of vascular symptoms in SSc

Functional, as well as structural arterial abnormalities play a central role from early on in SSc disease pathogenesis.[50] These abnormalities, although not exclusively, but predominantly affect the microvasculature, namely capillaries and arterioles.

2.2.1. Capillary abnormalities

The first location of functional vascular impairment and very often the first clinical sign of the disease is RP of the fingers. RP exerts triphasic pattern in SSc including pallor, numbness and pain of the fingers followed by cyanosis and hyperaemia of the acral skin caused by a disproportionate response of thermoregulatory, small and middle sized peripheral vessels to various stimuli such as cold or emotional stress. RP can be present for years before SSc develops. RP in SSc affects 95% of patients, and in 25-39% of patients is accompanied by digital ulcers and scarring. Furthermore, 11% of patients experience digital amputation as a severe complication of this symptom [19, 51-55].

The significance of nailfold or skin capillary pathology goes beyond classification, diagnosis or follow-up of local skin symptoms in SSc. Similarly to the ophthalmoscopic examination of the fundus revealing important information about systemic blood pressure control and atherosclerosis via visualization of ocular blood vessels and hypertensive retinopathy in the general population, nailfold video-capillaroscopy helps us detect the scale of pathological involvement of the SSc vasculature in general (video-capillaroscopy scores correlate well with clinical severity of SSc), and brought us closer to understanding SSc disease pathogenesis as well [19, 51-55].

The pathologic findings upon examinations of specimens from asymptomatic areas of patients with SSc presented direct evidence of general microvascular involvement as early as 1992 [56]. In addition to the above described capillary abnormalities, transmission electron microscopy reveals lamellation of the capillary basement membrane caused by amorphous material deposition possibly contributing to excessive capillary permeability and tissue edema; enlargement or swelling of endothelial cells and nuclei accompanied by early endothelial cell storage vesicle loss and rearrangement of the cytoskeleton, as well as diffuse perivascular and interstitial mast cell infiltration [57].

2.2.2. Small to medium size arteriopathy in SSc

Intimal proliferation and media hyperplasia, along with white blood cell accumulation and luminal obstruction resulting in an obliterative vasculopathy have been described in various types of muscular arteries such as coronary, peripheral and renal arteries in SSc. We have previously discussed cardiac as well as general vasculopathy. In the following section, pulmonary vascular involvement will be described briefly.

2.2.2.1.Vasculopathy of the lungs – pulmonary arterial hypertension (PAH)

Increased vasoconstriction and abnormal endothelial function result in a constant SMC contraction in pulmonary arterioles and a consequent rise in pulmonary arterial pressure (PAP). This complication is present in 7.9%-12% of patients with SSc. PAH is defined as a mean resting PAP of ≥25 mmHg with a pulmonary capillary wedge pressure ≤15 mmHg. N-terminal of B-type natriuretic peptide (NT-pro-BNP) levels >395 pg/mL, tricuspid gradient ≥ 45 mmHg on echocardiogram and diffusion capacity (DC) ≤55% as determined by DLCO help screening for PAH in SSc [58]. Since survival rates, cardiac index and therapeutic response of SSc-related PAH are much poorer than in iPAH[59] , it is suspected that either myocardiac fibrosis, inflammation and scarring or cardiac microvasculopathy compromises the ability of the right ventricle to compensate for pressure overload in SSc [60].

2.3. Vascular assessment in SSc

2.3.1. Use of vascular imaging techniques in SSc

Kerekes et al [61] have recently published a comprehensive review on validated methods for vascular assessment in autoimmune rheumatologic diseases. Hereby we only present a table about assessment of general vascular involvement and atherosclerotic burden in SSc (**Table 2**). Subsequently, assessment of endothelial function, arterial stiffness and ccIMT in SSc will be briefly discussed.

Table 2. Overview of imaging techniques of general vascular involvement in SSc [61].

Objective	Imaging technique
Assessment of microvascular function	Nailfold video capillaroscopy (noninvasive)
	Digital angiography (invasive)
Assessment of general vascular function	Pulse‑wave analysis
	Augmentation index
Evaluation of general subclinical atherosclerotic burden	Arterial stiffness parameters
	Measurement of local arterial elasticity and/or stiffness
	Pulse‑wave velocity (by mechanotransducers, applanation tonometry, ultrasonography or oscillometric methods)
	Common carotid intima–media thickness (ccIMT) with carotid plaque analysis
	Determination of coronary calcium content
	Evaluation of retinal vasculature
Evaluation of subclinical endorgan atherosclerosis	Ankle–brachial index (ABI, non-invasive)
	Intravascular ultrasonography (invasive)

2.3.1.1. Assessment of endothelial dysfunction in SSc

As previously mentioned, endothelial dysfunction is a consequence of some noxious impulse on the normal blood vessel and results in various molecular and structural, as well as cellular changes in the endothelium leading to functional changes. Of these, most easily assessable is the endothelial response to physical or chemical stimuli, such as shear stress, endogenous or exogenous vasoactive molecules, etc. prompting vasodilation, the degree of which is dependent on endothelial NO bioavailability. Indeed, currently validated methods for detecting endothelial dysfunction all operate with this phenomenon, see [61] for details**.**

The most widely prevailing method to assess endothelial function in SSc is the flow-mediated dilatation of the brachial artery (FMD), provoked by postocclusive reactive hyperemia. Normal value, or cut-off values of FMD may depend on the investigated disease and characteristics of the patients such as age and gender. FMD >4.5 % was proposed as a normal value in the general population by Schroeder et al [58], whilst an FMD <8.1% was associated with PAD [61-63]. Presently, there are no recommended cut-off values for FMD in SSc. The majority of FMD results in SSc vary between 2.1%-4.8% [62, 64-68], which correspond to impaired endothelial function. Reports suggesting decreased FMD in SSc [65, 67, 69-72] outnumber those reporting no significant

change compared to controls [64, 66, 68], supporting the findings of decreased FMD as a sign of endothelial dysfunction in this disease.

2.3.1.2. Definition and assessment of arterial stiffness

The term arterial stiffness refers to the decreased elasticity or abnormal stiffening of the conduit arteries. There are two most commonly used parameters to describe systemic arterial stiffness: pulse wave velocity (PWV) of the central elastic arteries, and augmentation index (AIx), which is a parameter characterising the pulse wave and its reflections from the periphery.

PWV is a parameter dependent on the elasticity of the central conduit arteries alone, thus it is more easily used for research or clinical purposes than AIx. Validated semi-automated methods for PWV and AIx assessment include Complior™ (Artech Medical, Pantin, France), SphigmoCor® (Atcor Medical, West Ryde, Australia) and the Arteriograph™ system (TensioMed, Budapest, Hungary) [61]. All these methods correlate with invasive techniques used to assess pulse wave analysis. The gold standard of PWV measurements is carotid-femoral PWV (c-fPWV), which has been shown to correlate with CV endpoints, and correlate with age in large epidemiologic cohorts, such as the classical Rotterdam study [73]. PWV has also been studied in SSc to determine if a correlation between clinical outcome or vascular symptoms can be established in this disease. A majority of studies indicate elevated PWV in SSc patients [64, 70, 74], however, several studies, although some with case numbers as low as 10 SSc patients, demonstrate no significant difference in the arterial stiffness parameters of SSc patients versus controls [66, 75, 76]. A unique distribution of arteries with abnormal stiffness was suggested by the findings of Liu et al , who reported increased local arterial stiffness parameters of the forearm and arm but normal systemic (aortic, femoral, lower limb) arterial stiffness parameters in a controlled study of patients with SSc. Although the reason for this is unclear, it seems plausible that the anatomical vicinity of pathologically involved SSc skin of the forearm or neck might permit neural signals, transmigration of inflammatory cells or circulation of biochemical signals promoting vascular changes in the area. Interestingly, increased arterial stiffness of the pulmonary circulation is also detectable and may indicate pulmonary vascular disease [77, 78], although these physiologic observations are yet to be studied in the clinical context. Possible additional techniques to assess c-fPWV includes ultrasound measurements [79], because the time delay between pulse pressure signals can be accurately measured by the foot-to-foot method. The calculation of the distance between the two measurement points, such as the carotid-femoral distance is much less accurate.

The relevance of PWV and AIx measurements in the clinical setting is limited both in the general population as well as in SSc by three factors including the lack of reliable cut-off or reference values; differences in semi-automated or manual measurement results and the need to maintain standardized conditions in order to rule out measurement bias [79-83]. Ultrasound-based methods to assess PWV are even less prevailing due to lack of invasive validation, inaccuracy in measurement of distance and need of ultrasound expertise. Accordingly, current ACC/AHA guidelines on CV risk assessment do not recommend these methods for clinical use, only for research purposes[84] .

2.3.1.3. Carotid intima-media thickness: a marker of overt atherosclerosis

Carotid arteries are an important and easily accessible example of the elastic arteries of the human body. Measurement of common carotid artery intima-media thickness (ccIMT, the distance between the first and second echogen lines starting from the lumen on a high-resolution B-mode ultrasound image) conveys almost identical results with pathologic and histologic examination of carotid wall thickness [84, 85]. Importantly, ccIMT below a threshold of 1 mm is considered a sign of general, rather than local atherosclerosis, and increased ccIMT compared to healthy controls has been shown to convey an increased risk of CV, cerebrovascular events as well as PAD [86], thus reflecting the total atherosclerotic burden.

According to a recently conducted metaanalysis by Au et al , ccIMT examinations indicate presence of early carotid atherosclerosis in SSc compared to controls. Of note, there was marked heterogeneity in previously conducted results of the ccIMT studies [36, 38, 66, 69, 71, 78, 87-89], however, adjustment of data for disease duration and age decreased heterogeneity substantially. Possible explanations for conflicting results in case of arterial stiffness or FMD may include methodological issues, whereas in case of ccIMT assessments, differences in ccIMT depending on disease subtypes and duration, chronically administered medications and selection of controls may contribute to difficultly comparable study results.

2.3.1.4. Ankle-brachial index (ABI): assessment of peripheral atherosclerosis

ABI is a simple examination to screen for subclinical vascular stenoses, hence atherosclerosis or PAD of the lower extremity. Since PAD is associated with both CV (increased risk of MI) and cerebrovascular disease (e.g. stroke) in the general population, patients with PAD have been proposed as candidates for secondary prevention [61]. Since stenoses of relevant arteries usually

develop gradually in generalized atherosclerosis, functional collaterals have time to develop. Due to these collaterals, however, even patients with flow-limiting stenoses can spend a significant time asymptomatically, in which phase screening with ABI measurement helps identify silent stenoses of the lower extremity arteries. Technically, the examination is carried out in a supine position after a resting period. Systolic blood pressure of the brachial artery, the anterior tibial artery and the posterior tibial artery (cuffs inflated over the lower calves) of both sides is determined using a continuous wave Doppler probe. Variations in methodology exist, but most accepted is the division of the highest blood pressure of both lower limbs by the blood pressure of the brachial artery. This ratio is defined as the ABI, with values between 0.9-1.3 considered as normal. Values below 0.9 signify stenosis, whereas values below 0.4 usually represent critical ischemia. According to some studies, a cut-off value of a systolic blood pressure of ≤60 mmHg better reflects critical limb ischemia [61, 90]. Interestingly, not only values below 0.9, but also above 1.3 indicate increased cardiovascular risk [91, 92]. The explanation is that severe arterial medial calcification leads to incompressible arteries, especially in patients with diabetes, in which case the blood pressure values measured over the ATP and ATA are higher than usual. ABI is a method of moderate sensitivity (around 70%), which can be improved by post-exercise testing or by using the lowest of the 2 lower extremity blood pressure measurements.

ABI assessment in SSc has conveyed conflicting results so far, with studies reporting normal ABI in SSc [91-95] outnumbering those, which found mildly decreased ABI [96] It seems, however, that abnormal ABI is present at least in some subsets of limited SSc patients even though it does not appear to be a general feature of SSc.

2.3.2. Biomarkers of vascular involvement in SSc

2.3.2.1. Overview of biomarkers

Estimating 10-year CV risk in rheumatic diseases and specifically SSc by the same method as that of the general population, such as the Framingham risk score or the SCORE chart, does not represent the real prognosis of SSc patients, due to various disease-specific, often CV, conditions. Thus, constant efforts have been made by researchers in order to identify novel reliable tools and markers to understand pathogenesis, help diagnosis or disease subset classification (e.g. the relationship of anti-centromere antibodies and PAH,), refine risk stratification for organ involvement (e.g. for development of PAH, vascular involvement or fibrosis), or to evaluate SSc patients' response to therapy [97, 98]. In **Table 3,** some biomarkers related to vascular involvement and

therapeutic response in SSc are listed. Subsequently, the role of vWF antigen and CRP as biomarkers in SSc will be summarized.

2.3.2.2. Von Willebrand factor (vWF) antigen

A member of the coagulation-haemostasis system, and a marker asscociated with endothelial function, vWF levels have been found to be elevated in SSc and to be associated with endothelial dysfunction, ILD, as well as disease activity in SSc [98]. The molecular mechanism suggested to account for this is reduced cleavage of vWF by the lower levels of enzyme ADAMTS-13 in SSc, which thus might have a role in maintaining elevated vWF levels in SSc. However, according to a metaanalysis of results to date, vWF is probably unsuitable for evaluation of response to treatment in SSc [93].

Table 3. Some CV biomarkers and their use in SSc. (For abbreviations please see page 2.)

Marker of:	Name	Significance	Reference
Organ involvement	NT-proBNP >125 pg/ml ET-1 Hyponatraemia	Diagnosis of PAH Therapeutic response Severe PAH	[98-100],[101],[102], [19], [76] [59, 103]
Vascular involvement and endothelial dysfunction	Homocystein, MTHFR vWF low circulating EPC count E-selectin,ICAM,VCAM-1	Endothelial dysf.+macrovasc. Endothelial dysfunction CV events Vasculopathy	[104], [105], [106] [98] [107, 108], [25] [109]
Angiogenesis	VEGF PlDGF ADMA	Angiogenesis, early disease Angiogenic factor Oxidative stress, ↑ CV risk	[109] [25] [110-112],[113], [114]
Immuno-inflammatory processes	HMGB-1, AGE CRP, IL-6 Sr-IL-2, Sr-TNF-α	Related to SSc severity (RSS) Dis. activity, severity, prognosis Skin /pulmonary fibrosis severity/progression	[30], [31] [115], [116], [117] [98]
Atherosclerosis	Adiponectin PAI-1 AECA	Associated with skin fibrosis, lung disease in SSc Linked to carotid atherosclerosis in SSc; Advanced capillary and cardiac involvement	[118, 119] [120] [121], [122-124]

2.3.2.3. The role of CRP and IL-6 in SSc

Metaanalyses and reviews show that the acute phase reactant C-reactive protein (CRP), as well as the major cytokine responsible for CRP release, IL-6, are both important in SSc pathogenesis and clinical manifestations [117]. There are single nucleotide polymorphisms and gene-gene interactions

which affect SSc predisposition, manifestation and expression of IL-6. Studies of animal models demonstrate that both IL-6 and IL-6 trans-signalling are involved in the pathogenesis of SSc. IL-6 is regulated by T and B cells of altered function during disease development. Fibroblasts, T/B cells, monocytes, macrophages, dendritic cells and endothelial cells all participate in IL-6 expression and their crosstalk results in tissue sclerosis. Up-regulation of serum IL-6 and CRP levels (CRP >8mg/l is present in approximately 25.7% of all cases) are evident in SSc patients and associated with disease activity, severity (mRSS, HAQ, serum creatinine and decreased lung capacity), disability, poor outcome and reduced survival. Albeit IL-6 is a weaker biomarker than CRP due to its shorter halflife in serum [116], targeted IL-6 therapy in SSc has occurred in small cases series and there are ongoing controlled clinical trials addressing anti-IL-6 therapy [117]. Altogether these data suggest CRP is a nonspecific but important marker in the clinical care of SSc patients.

2.4. Therapeutic options for the SSc-affected vasculature: the role of statins

2.4.1. Description of widely used drugs

Based on evidence and clinical experience of the European League Against Rheumatism (EULAR), currently recommended therapy of SSc stands on three bases: immunosuppressive therapies, applied mainly in early, severe, rapidly progressing, diffuse forms of the disease, particularly in patients with ILD; anti-fibrotic therapy and vasculoprotection. This latter group includes dihydropyridine-type calcium-channel blockers, iv. prostacyclin for digital vascular symptoms and ulcers, as well as ET-1 receptor antagonists (bosentan, macitentan), PDE5 inhibitors (sildenafil, tadalafil) and the prostanoid epoprosterenol for therapy of PAH. ACE-inhibitors have been recommended in SSc, mainly due to their renoprotective effects [125]. For patients with RA, the rheumatic disease with the greatest atherosclerotic burden, but also for spondyloarthritis patients, EULAR recommends administration of statin therapy, when necessary, to decrease CV risk [126]. However, in SSc, no such recommendation exists today. An increasing array of possible vascular targets are shown on **Figure 2**.

2.4.2. The pleiotropic effects of statins

Since the second half of the 1990s, increasing body of evidence has accumulated to support that statins, originally used as lipid-lowering drugs via HMG-CoA-reductase inhibition, may exert multiple anti-atherogenic effects. Statins may reduce arterial stiffness [127], improve endothelial

function [128] by increasing NO bioavailability via eNOS [129, 130] and may convey antioxidant, anti-inflammatory [131] or potentially immunomodulating effects [132]. In addition, stabilization of the atherosclerotic plaque, decreased vascular SMC migration and proliferation, and inhibition of platelet aggregation have been highlighted as favourable non-lipid effects of statins [133]. Therefore, it is logical that several studies assessed the potential of statins on arterial stiffness, subclinical signs of atherosclerosis or arterial calcification, in asymptomatic patients or those with atherosclerosis or a scope of diseases involving vascular pathology. All these effects lead to favourable CV outcome in statin-treated patients [122-128].

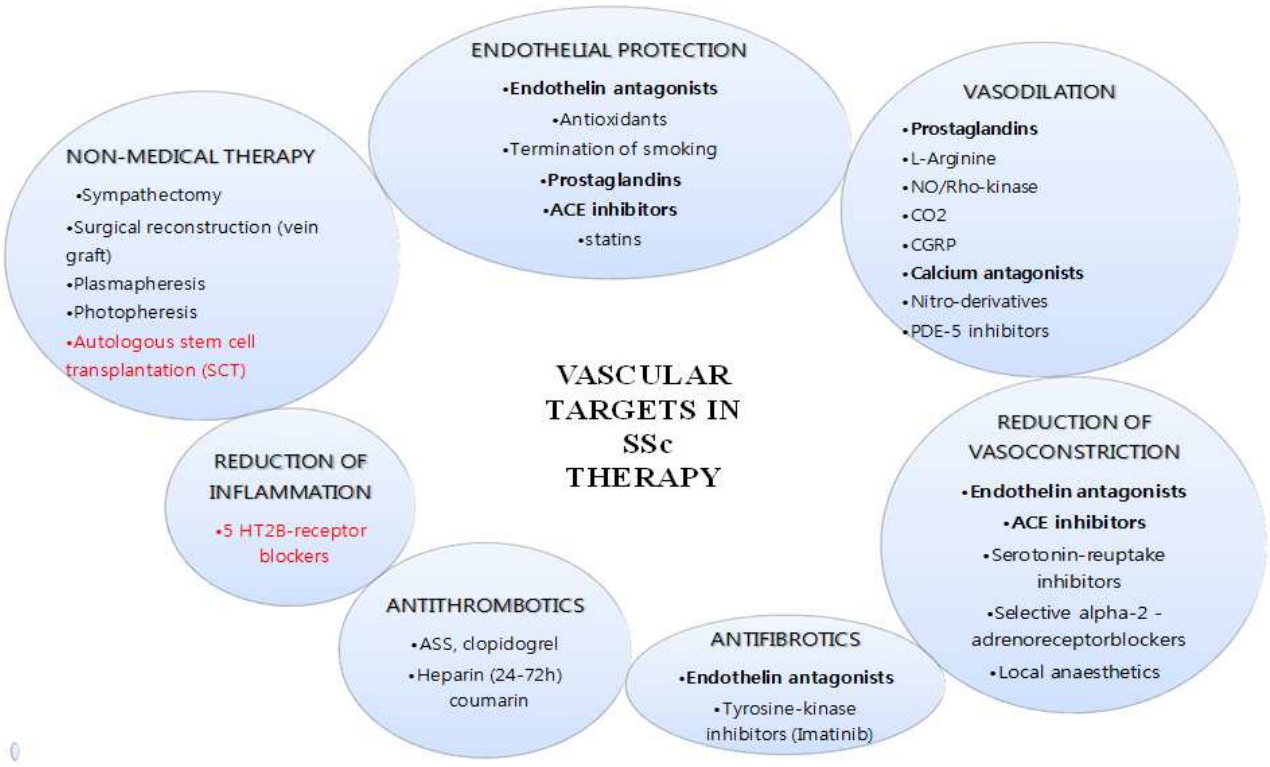

Figure 2. Overview of current vascular targets in the therapy of SSc. Modified after [12]. Drugs in bold were recommended by EULAR for management of SSc [186].

In order to understand the heterogeneity of effects exerted by statins, we must imagine the biochemical process influenced by statins and the exact structure of these drugs as well as their mechanism of binding to HMG-CoA reductase. The group of statins are all similar in structure in having a HMG-like moiety, linked covalently to a more-or less hydrophobic part of the statin molecule. Statins exert their effect by binding to the active site with the HMG-like part, and by sterically inhibiting substrate binding to HMG-CoA reductase, a phenomenon demonstrated by 3D

X-ray crystallographic imaging by Istvan et al [134]. Rosuvastatin is the statin that exhibits the greatest number of binding interactions with HMG-CoA-reductase and the lowest median inhibiting concentration, thus greatest efficacy, among all statins, explaining why this is the most potent reductor of cholesterol-synthesis *in vivo*. Pleiotropic effects of statins are likely to result from changes downstream to HMG-CoA reductase [135-137]. Multiple molecular mechanisms have been proposed to account for the non-lipid-lowering effects of statins. Most important are upregulation of endothelial NO synthase, inhibition of NADPH oxidase and thus a decrease in ROS production (**Figure 3**).

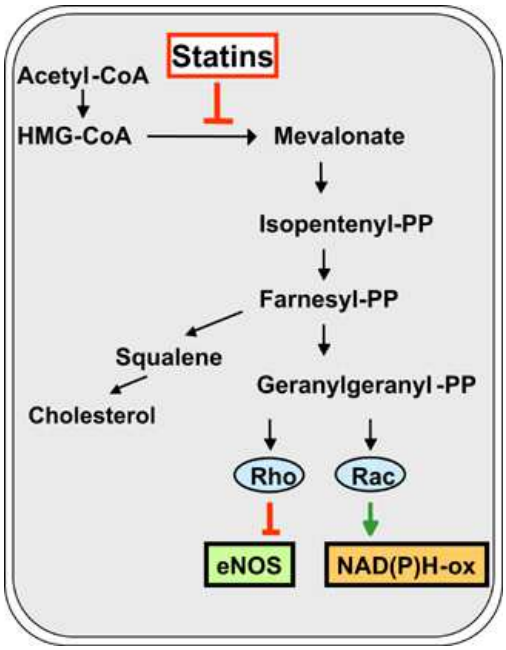

Figure 3. Inhibition of the mevalonate pathway of cholesterol biosynthesis by statins.
From: Endres et al [136].

The mevalonate pathway is involved in synthethizing the isoprenoids farnesyl- and geranylgeranyl-pirophosphate, necessary for small GTP-binding proteins Rho and Rac for their activation via farnesylation and translocation from the inactive cytosolic state to the cell membrane. Since Rho inhibits endothelial NO synthase, in absence of farnesylation, an increased eNOS activity will be observed. Similarly, since Rac is part of the NADPH oxidase complex required for ROS production, impaired farnesylation due to statin therapy will result in a decrease in the concentration of reactive oxigen species [124].

Importantly, median inhibiting concentration of HMG-CoA reductase of statins is measured in hepatocytes, whereas their pleiotropic effects may be dependent on their transport through the various cell membranes (endothelial, macrophages, etc.) determined by their lipophilicity. Thus it is

important to note, that rosuvastatin, the most efficient antilipid effector is the least lipophilic, hence its transport through the cell membrane is much more difficult than that of atorvastatin.

Additionally, statin effects also include reduction in CRP, IL-6, TNF-α and NF-κβ levels, interference with the blood coagulation cascade by decreasing coagulation activity and platelet aggregation, as well as normalisation of symphathetic outflow [138]. Anti-inflammatory effects of statins are partly explained by a recently described non-mevalonate pathway effect of statins, for example, by binding to a novel regulatory integrin site, statins are able to block adhesion and costimulation of leukocytes [139].

In the past decades, an increasing number of studies addressing either the effects of statins in SSc [137, 140-145], or the pleiotropic effects of rosuvastatin in reduction of CV risk in the general population have come to light [146-150]. Given a highly efficient statin with prior controlled studies indicating its protective effect in increased risk as well as intermediate-risk, symptom-free individuals, we decided to assess CV effects of rosuvastatin in a selected group of patients with SSc.

3. OBJECTIVES

1. To assess the markers of endothelial function, namely flow-mediated vasodilation (FMD) and nitrate-mediated vasodilation (NMD) as well as to look for clinical signs of subclinical atherosclerosis via measurement of ccIMT in a cohort of patients with SSc compared with individually matched healthy controls.

2. To search for a relationship between the above parameters and age, SSc duration, organ involvement or autoantibody positivity.

3. To examine arterial stiffness, a risk factor for CV disease and a sign of large vessel involvement, in patients with SSc and compare these values to healthy controls determining PWV and AIx by an automated oscillometric method.

4. To compare PWV and AIx results in limited and diffuse disease subsets and to investigate age-dependency of the above parameters as well as their correlation with disease duration in SSc.

5. To investigate the effect of 6-month rosuvastatin treatment on different macrovascular parameters: the endothelial functional marker FMD, arterial stiffness examined by PWV, peripheral arterial disease screened by ankle-brachial index (ABI) and ccIMT or presence of a carotid plaque as a sign of general atherosclerosis. In addition, we assessed the possible effects of 6 month rosuvastatin therapy on microvascular function of the skin by measuring forearm cutaneous blood flow by Laser Doppler perfusion monitoring (LDPM) before and after treatment.

6. To determine effects of rosuvastatin on serum inflammatory markers (CRP, ESR), the endothelial marker vWF antigen, complement (C3, C4) and immune complex (IC) levels, as well as basic laboratory parameters (serum lipid levels, renal function, liver enzymes, complete blood count) preceding and following 6-month rosuvastatin treatment in patients with SSc.

7. To determine and characterize cardiac involvement in SSc by resting conventional and pulsed wave tissue Doppler echocardiography, resting ECG and 24-hour Holter ECG monitoring. To follow basic echocardiographic parameters such as baseline and post-treatment left ventricular ejection fraction (EF), indices of diastolic function, right ventricular function, and right ventricular pressure as well as signs of valvulopathy preceding and following 6-month rosuvastatin therapy in SSc patients. Our goal was to search for a relation between the presence of arrhythmias and

echocardiographic abnormalities in SSc. In addition, we sought to follow-up physical fitness by repeated 6-minute walk tests before and after rosuvastatin therapy.

4. PATIENTS AND METHODS

4.1. Patients and study protocols

4.1.1. Endothelial function and ccIMT in SSc

In the first study, 29 randomly selected SSc patients (female:male ratio=25:4; mean age ±S.D.: 51.8±10 years [range: 31-69 years]) and 29 healthy controls including hospital visitors and employees (female:male ratio=23:6, mean age 49.3±6 years [range: 25-69 years]) were included after screening. The diagnosis of SSc was established according to the SSc criteria described in 1980 by the American College of Rheumatology (ACR) [1]. A written consent form from each participant was obtained. The average disease duration of SSc patients was 9.43±3.78 years (range: 2-23 years) as calculated from the onset of RP or other acral symptoms of the fingers, such as numbness, acrosclerosis, or swelling. The majority of patients (n=19) belonged to the lcSSc subset, whereas 10 patients suffered from the dcSSc form of the disease.

Organ involvement was screened by the following methods: pulmonary fibrosis by HRCT and pulmonary function tests, esophageal involvement by barium swallow test, cardiac involvement by ECG and echocardiography (an estimated right ventricular pressure >35 mmHg was defined as PAH), and renal disease screened by clinical symptoms and laboratory parameters of renal function, completed by biopsy, when necessary. In both patients and contro75ls, traditional CV risk factors including age, body mass index (BMI), plasma lipid profile, as well as systolic and diastolic blood pressure were determined. Each patient and a corresponding control subject were matched according to age and risk factor status. Considering that we wished to study subclinical surrogate markers of atherosclerosis as well as endothelial dysfunction, exclusion criteria included existing CV disease, diabetes mellitus, cigarette smoking, obesity (BMI≥30), vasculitis, acute or chronic infection as well as renal failure (defined as serum creatinine levels >117 μmol/l).

The study protocol was the following: all patients and controls were fasting and had been asked to suspend alcohol, tobacco, antioxidant and vasoactive drug intake for at least 24 hours prior to the assessments. On the morning of the vascular examinations, a fasting blood sample was drawn for renal functional parameters and serum lipid profile and participants underwent the common carotid intima-media thickness (ccIMT), brachial artery flow-mediated dilation (FMD) and nitrate mediated dilation (NMD) examinations as detailed below.

4.1.2. AIx and PWV in SSc

In the second study assessing AIx and PWV in SSc, a total of 46 consecutive patients of female prepondarance, appearing for regular checkup at our clinic were screened, and 40 patients (with a female to male ratio of 36:4) were found eligible for the study. Inclusion and exclusion criteria were identical to the previous study. The only difference between the two studies was the exclusion of patients with constant arrhythmias in the present study due to methodological reasons. A written consent form from each participant as well as an Institutional Review Board approval was obtained for the study. Patients all fulfilled the ACR criteria for SSc [1]. The mean age of eligible patients was 58.0 ± 12.3 years (range: 33–81 years), who dominantly suffered from the lcSSc form of the diseae (31/40 patients). For comparison, we studied 35, age- and sex- matched healthy controls (female:male ratio=32:3, mean age 53.0 ± 10.5 years [range: 30–77 years]). The average disease duration in the patient group was 12.5 ± 6.7 years (range: 1–27 years). Traditional risk factors for CV disease, such as age, BMI, lipid levels, as well as systolic and diastolic blood pressure were assessed and differences between the patient and control group were found non-significant (data not shown).

On the morning of the vascular examinations, both patients and controls underwent a physical examination to exclude acute infection or arrhythmias and to determine baseline blood pressure and BMI values. Subsequently, fasting blood samples were drawn for serum LDL-, HDL-, total cholesterol and trigliceride levels as well as serum creatinine. Following a resting period in a quiet study room, arterial stiffness parameters AIx and PWV were determined.

4.1.3. Effects of rosuvastatin on cardiovascular parameters and biomarkers in SSc

SSc patients arriving for regular checkup to our institution were randomly screened for inclusion and exclusion criteria as detailed below. Diagnosis of SSc had previously been established according to the ACR criteria[1]. Following screening, 28 patients were found eligible for the study (female:male ratio=25:4, median age: 60.4±11 years [range: 34-83 years]). The majority of patients (25/29) had intermediate disease duration or late disease, thus mean disease duration was 13.6±7.7 years (range: 2-30 years). Altogether 75% of patients suffered from lcSSc, while 25% had the diffuse form of the disease.

Clinical manifestations in order of decreasing prevalence among patients were RP (96%), pulmonary involvement including ILD (63%) or mild PAH (7%), distal skin manifestations including sclerosis and ulcers (68%), GI manifestations (54%), proximal skin involvement (32%), known cardiac manifestations (25%), sicca syndrome (10%), renal involvement (4%). In 6 patients (22%), SSc overlapped with another CTD, namely dermato-polymyositis (2 patients), SLE (1

patient) or RA (3 patients). The patients' medications are listed in **Table 4**. All recruited patients were non-smokers and their mean BMI was 23.1 ± 4.1 kg/m2.

Table 4. Pharmacological therapy of SSc patients at inclusion (Study 3)

Medication	Number of patients	%
ARB/ACE inhibitors	21	75
Calcium channel blockers	16	57
Beta blockers	8	29
Corticosteroids	9	32
Pentoxifylline	23	82
Nitroglycerine	4	14
Vitamin E	8	29
Platelet Aggregation Inhibitors	16	57
H2-receptor blockers or proton-pump inhibitors	14	50
Immunosuppressive agents	3	11
NSAIDs	5	18
Bisphosphonates	3	11
Others (diuretics, tramadol, benzodiazepines, bronchodilators)	14	50

Inclusion criteria included presence of microvascular symptoms including new digital ulcers, active RP despite ongoing therapy, and informed consent of patients. Exclusion criteria included hyperglycemia, acute systemic infection, uncontrolled hypertension, carotid sinus hyperesthesia, permanent atrial fibrillation, an EF<50% as determined by echocardiography, severe PAH, smoking, active ulcers at any of the measurement site as well as vasoactive drug treatment such as prostanoids, or lipid-lowering drug treatment in the previous 6 months prior to screening, or patients requiring frequent therapeutic adjustments. The study was performed according to the Declaration of Helsinki under the auspices of the University of Debrecen Institutional Review Board.

All examinations, including laboratory analyses as well as the functional and structural vascular assessments described below, were performed on two occasions, directly before and after the rosuvastatin treatment period. On the day of vascular assessments, a blood samples were drawn between 7 and 8 a.m. after an 8-hour fasting period. Samples were stored at room temperature and analyses (of serum inflammatory markers (CRP, ESR), the endothelial marker vWF antigen, complement (C3, C4) and immune complex (IC) levels, as well as basic laboratory parameters: serum lipid levels, renal function, liver enzymes, complete blood count) were performed within 2 hours. vWF antigen samples were stored on ice until analysis. Alcohol, caffeine consumption, as

well as administration of vasoactive and antioxidant drugs were suspended for 24 hours prior to examination. Vascular assessments were carried out in a quiet, darkened study room with a steady temperature (22±1° C) following a 10-minute resting period in a supine position according to the recommendations of Laurent et al [13] and each vascular assessment was performed by the same examiner (OT). Patients underwent detailed echocardiography, ccIMT measurements, ultrasound-based aorto-femoral and carotid-femoral PWV measurements, brachial artery FMD assessment, ankle-brachial index and post-occlusive reactive hyperemia testing during laser Doppler forearm skin perfusion monitoring. In addition, a resting 12-lead ECG was obtained and 24-hour Holter ECG monitoring as well as a 6-minute walk test was performed. Following baseline measurements, each patient recieved 20 mg rosuvastatin per day for 6 months. All patients tolerated the drug well and we experienced no drop-outs. Measurements were repeated following 6 months under conditions identical to those of baseline assessments.

4.2. Vascular assessments

4.2.1. Brachial FMD and NMD

Flow-mediated dilatation (FMD) is a method for quantification of the vasodilation of the brachial artery by ultrasonography, following a standardized physical stimulus, usually post-occlusive reactive hyperaemia (PORH), during which we create a distal ischemia, induce vasodilation thus evoke shear stress, and test endothelium reactivity of the afferent artery to this shear stress. During brachial artery FMD testing, after a resting period and under standardized circumstances [151], the brachial artery is visualized at the antecubital fossa with a 7-15 Mhz transducer parallel to ECG gating. Resting brachial artery diameters are determined, then approximately 50 mmHg suprasystolic pressure is applied by inflation of the cuff for at least 4.5 minutes, keeping the assessed arterial segment in view constantly. This ischemic period results in a marked dilation of the resistance vessels. Upon cuff deflation, a rapid increase in blood flow occurs, resulting in vasodilation of the afferent artery with the maximum dilation occurring at about one minute after deflation. ECG-gated images are obtained for offline analysis for 3 minutes, and the diameters of maximum dilation are used to calculate FMD.

In order to assess endothelial function in the first study, brachial FMD and NMD were determined according to the 2002 guidelines by the American College of Cardiology [151] under standardized conditions [152]. Following a 30-minute resting period in room at 22±1°C, ultrasound

examinations were performed by a single, trained assessor with a HP Sonos 5500 ultrasound equipment and a 10 Mhz linear array transducer. Baseline brachial artery diameters (BAD_{basal}) were obtained about 4-7 cm-s proximally to the cubital fossa, taking into account individual anatomical variations. BAD measurements were repeated 5 times and averaged. Subsequently, a pneumatic cuff was inflated over the forearm to a suprasystolic pressure for a total interval of 4.5 minutes. Upon deflation, BAD, as well as maximum flow velocity, were again assessed for 90 seconds, and maximal arterial diameter (BAD_{max}) was determined. FMD was expressed as the percent change from the baseline value ((BAD_{max}-BAD_{basal})/BAD_{basal}x100).

NMD assessments were carried out after a 15 minute resting period as follows: baseline BAD were again obtained as described above. Thereafter, 400µg (1 spray exposition) of sublingual nitroglycerine was administered to the patient and changes in BAD were recorded constantly for 4 minutes. Maximal BAD values were used for estimation of NMD, again, as percent change from the baseline. Reproducibility of the method was assessed in our laboratory resulting in a variation coefficient of 5%, and an intraclass coefficient of 0.935, which is considered excellent. For Blond-Altman plots see [153].

4.2.2. ccIMT

ccIMT examination is carried out in a supine position with a high-resolution (at least 7 MHz) linear array transducer. The bulb and common carotid section of the artery is visualized and ECG-synchronized images are obtained at end-diastole via synchronization to the R wave on the electrocardiogram. Recommendations suggest performance of the examination only on the common carotid section and the far wall of the artery [154]. The advantage of ccIMT measurements in the clinical setting is a good reproducibility, but an intermediate sensitivity. Thus, ccIMT measurements should always be conducted parallel to plaque analysis [154].

In our studies, ccIMT assessments were carried out according to Kanters et al [155], in accordance with current guidelines[80, 151, 152]. Briefly, we visualized and screened both right and left common carotid arteries including the carotid bulb by the same duplex utrasound system we used for our FMD and NMD measurements (HP Sonos 5500, 10 MHz). Finally, ECG gated, end-diastolic (R synchronized) longitudinal sectional images of the common carotid artery were saved. Offline measurements were performed 1 cm proximal to the carotid bulb in the far wall of the artery using the leading edge method. ccIMT was defined as the distance between the first (intima-lumen) and second (media-adventitia border) echogenic lines starting from the lumen, an average of 10 measurements were used on both sides for ccIMT calculation, and ccIMT values were expressed in mm.

The intraobserver variability of ccIMT in our laboratory was excellent: the calculated variation (VC) and and intraclass coeffitients (ICC) were 4.2% and 0.98, respectively, indicating very good reproducibility.

4.2.3. AIx and PWV

The shape of the pressure waveform originating from the left ventricle (see **Figure 4.**) is influenced by the flexibility and distensibility of the aorta and its proximal branches as well as by reflection from anatomic branching points and changes in peripheral vascular resistance. The pulse wave is defined as summation of the pulse pressure wave originating from the heart and its reflections [61]. In a healthy vasculature, the reflected wave arrives in diastole and does not cause an additional afterload compared to the pulse pressure. If the reflected wave arrives early, in systole, it augments the original pulse pressure. AIx (%) is calculated as the ratio of the augmentation pressure and the pulse pressure, multiplied by 100. (**Figure 4**.)

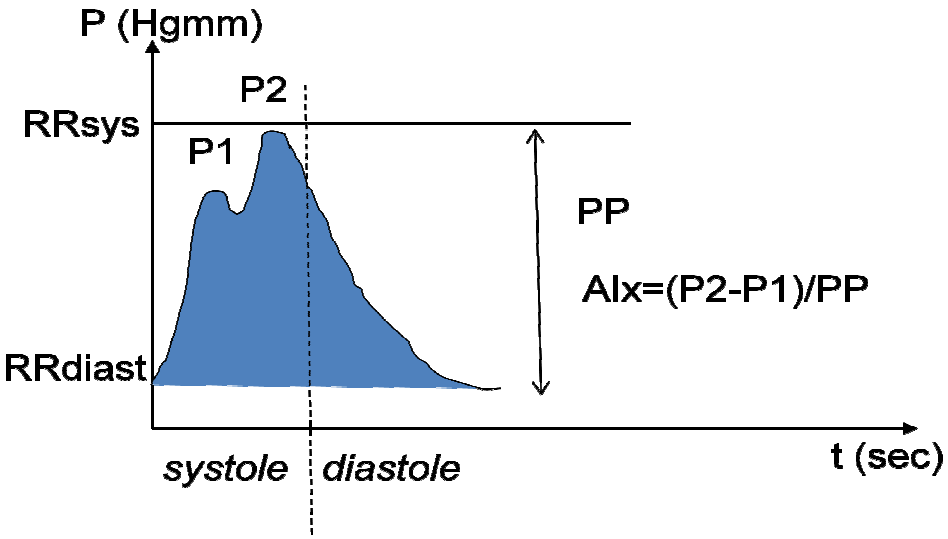

Figure 4. Augmentation index (AIx) of an abnormal pulse wave. The red curve indicates changes in arterial pressure over time. The pulse wave is constituted by an afferent pressure wave (its peak causes an inflection point, P1) and a reflected wave, evoking a late systolic peak (P2). ΔP: augmentation pressure (P2-P1), RRdiast: diastolic pressure, RRsys: systolic blood pressur, PP: pulse pressure (RRsys-RRdiast). AIx is a positive value if P2 arrives in systole (leading to increased systolic pressure and pulsatility, greater AIx indicates a more rigid vasculature). In healthy (elastic) vessels, the returning wave reflections arrive in diastole to the aortic root, thus do not increase pulse pressure (AIx will be of negative value).

In the second study, we assessed two parameters, PWV, the velocity of the propegating pulse wave in the central elastic arteries, and AIx, a value which expresses the ratio of augmented pressure compared to the pulse pressure characterizing arterial stiffness. Measurements were performed by

the arteriograph system (Tensiomed™ Ltd., Budapest, Hungary), which had previously been validated by comparison to the standard SphygmoCor and Complior systems [156], as well as to invasive measurements of arterial stiffness [157, 158]. Arteriograph measurements correlated well with the above methods [156-159]. During the examination, first a systemic blood pressure was automatically measured with a right arm cuff, subsequently a pressure waveform was obtained by the same cuff with an oscillometric method at 35 mmHg suprasystolic pressure. Thereafter, the curve was analysed automatically to calculate brachial and central AIx as well as PWV. PWV was calculated automatically by the Arteriograph system as the quotient of two times the distance between the jugular fossa and symphysis and RT S35 (reflection time at 35 mmHg suprasystolic pressure). Arteriograph uses the jugular fossa–symphysis distance as a surrogate for the length of the descending aorta between the aortic trunk and the bifurcation.

In the third study, arterial stiffness parameters were determined by an ultrasound-based method. Aorto-femoral PWV (a-fPWV) was measured on a HP Sonos 5500 ultrasound equipment as described by Bodnar et al [160]. Briefly, suprasternal (aorta pulsed wave Doppler signal, 2-4 Mhz phased array transducer) and femoral images (common femoral artery pulse Doppler signal at the level of the inguinal ligament, assessed by a 5-10 MHz linear array transducer) were obtained during simultaneus ECG recording. Pulsed Doppler analysis with 5 mm sample volume at 150 mm/s sweep speed was performed over the beginning of the aorta descendens and common femoral artery over two breathing cycles (10-12 cardiac cycles).

The distance between the suprasternal notch and the aortic measuring site (d1) as well as distance between the suprasternal notch and the femoral measuring site (d2) was measured, and time delay between the R wave and feet of the ECG gated aortic and femoral signals was used as pulse transit time. a-fPWV was calculated as a ratio of (d2-d1)/(t2-t1) and expressed in m/s. c-fPWV assessments were performed similarly, with minor differences. The two measuring points of c-fPWV were the left common carotid artery (1 cm proximal to the the carotid bulb) and the right common femoral artery (see a-f PWV measurement). Pulse transit times were again calculated in relation to the ECG signal using the foot-to-foot method and the distance between the two sites was defined as the difference between the jugular notch jugulum-carotid measurement point and the jugular notch-femoral measurement point distance. c-fPWV was again the quotient of the distance and the transit time [79, 161, 162].

Reproducibility of arterial stiffness measurement results was ensured by maintaining constant, neutral (22±1°C) room temperature, performance of measurements in a supine position following a 10-minute resting period, suspension of tobacco and caffeine consumption for at least 10 hours, adhering to standard recommendations [82, 83].

4.2.4. Ankle-brachial index

Ankle-brachial index (ABI) was assessed corresponding to inter-society consensus guidelines [163, 164] to screen peripheral arterial disease. A 10 to 12 cm sphygmomanometer cuff was placed just above the ankle of the patients and a handheld CW Doppler instrument (Vasodop 8 MHz, MediCAD Ltd, Miskolc, Hungary) was used to measure the systolic pressure of the posterior tibial and dorsal pedal artery of each leg as well as the brachial artery systolic pressure. The higher of the lower limb artery pressures was divided by the higher brachial systolic blood pressure value to form the ABI.

4.2.5. Laser Doppler Perfusion Monitoring

Microvascular skin perfusion was assessed by post-occlusive reactive hyperemia testing during Laser Doppler Perfusion Monitoring (LDPM). During this examination, a laser beam (with a wavelength of 780 nm) penetrates 1-1.5 mm deep into the skin, while a fraction of the light is scattered back by moving blood cells resulting in a frequency shift according to the Doppler principle. Therefore, a signal, proportional to tissue perfusion, is generated. Physical stimuli, such as heat or PORH, or biochemical provocation, such as vasoactive agents applied by iontophoresis, allow for testing of skin reactivity [165]. We applied a standard laser probe (PF 408) fixed in a straight probe holder (PH 08) of a Periflux PF 4001 LD flowmeter (Perimed AB, Järfällä, Sweden). A computer linked to the LD apparatus displayed recordings and saved the information for further offline analysis (Perisoft for Windows, Ver. 2.5.5 software, Perimed AB, Järfällä.) Measurements were carried out with the patients resting in supine position. The probes were placed on the volar side of the mid-forearm skin, avoiding superficial subcutaneus blood vessels, and basal blood flow was recorded for eight minutes. Afterwards, during continuous recording of skin blood flow, the upper arm obstruction was applied for 3 minutes (by inflation of a 10 to 12 cm sphygmomanometer cuff to 50 mmHg suprasystolic pressure). Subsequently, the cuff was suddenly deflated and forearm skin blood flow was further recorded for five minutes. By the end of this period, blood flow returned to baseline (**Figure 5.**)

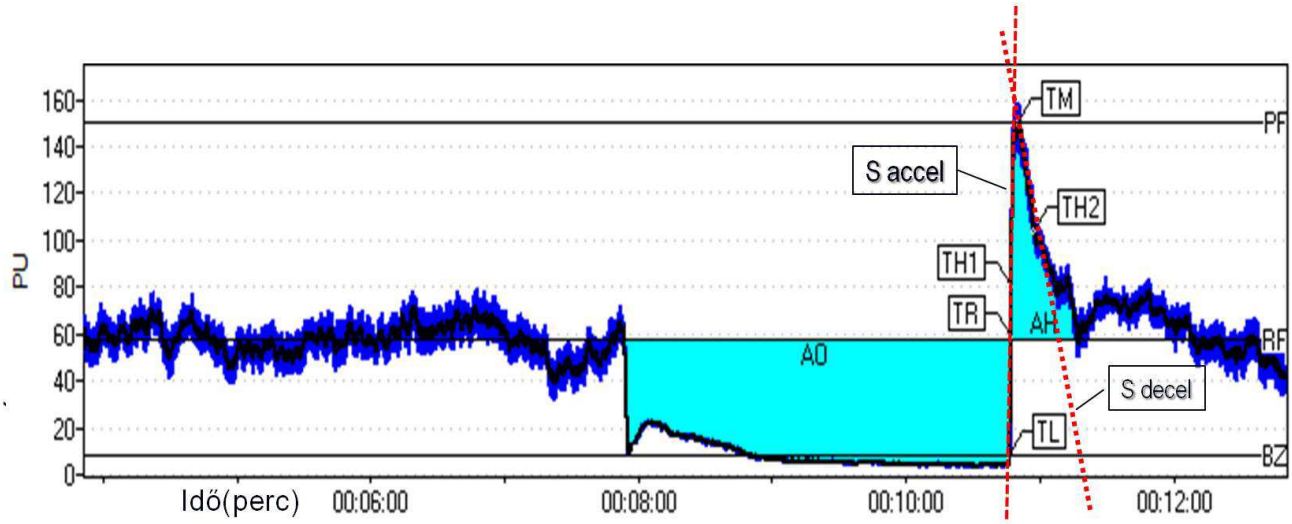

Figure 5. Laser Doppler perfusion curve during PORH testing. Following 8 minutes of recording (resting flow), we applied 3 minutes suprasystolic pressure to reach biological zero flow (AO). At 11:00, following rapid deflation, flow velocities rose to peak flow (PF), then slowly decreased to return to baseline. PU: perfusion units, AO: occlusion area, RF: resting flow, BZ: biological zero flow, TL: time to latency, TR: time to recovery, TH1: time to half before hyperemia, S_{accel}: slope of the accelerating perfusion curve between BZ and PF, TM: time to maximum flow, TH2: time to half recovery, S_{decel}: slope of the decelerating curve between peak flow and resting flow. AH: hyperemic area.

Blood flow (basal, peak, biological zero) was expressed in arbitrary perfusion units (PU) and relative changes compared to basal values were assessed. Time to half before hyperaemia (TH1, seconds), time to maximum flow (TM, seconds) and time to half recovery (TH2, seconds) were analysed. Additionally, the occlusion areas (AO), hyperaemic areas under curve (AH, PU * seconds), as well as hyperemia repayment (AH/AO) were determined. We assessed the slope of the curve as it reached maximum perfusion after cuff release (acceleration slope) and upon return to resting skin flow (deceleration slope). To our current knowledge, LDPM together with PORH provocation is a sensitive method to assess endothelial dysfunction of the skin, however, only the time curve of the PORH response is considered reliable due to reproducibility issues[166].

4.3. Cardiac assessments

4.3.1. Standard echocardiography and tissue Doppler myocardial imaging

Echocardiography was performed by a skilled echocardiographer under the supervision of a cardiologist before and after therapy, as described by D'Andrea et al [167]. Two-dimensional (parasternal long- and short axis views, as well as apical 3 chamber and four chamber views and assessment of the portal vein), M-mode and Doppler imaging were performed and basic

echocardiographic parameters were determined with patients resting in left decubitus position. Images obtained by a 2-4 MHz phased array transducer of a HP Sonos 5500 ultrasound equipment were analysed over 3 cardiac cycles, and the average of three measurements was used. Septal and lateral wall thickness was analyzed at end-diastole in the parasternal short-axis view. Left ventricular mass, (LVM), indexed for height, was calculated according to Devereux et al [168] by the following formula:

$LVM=1.04\ ((LVID+PWT+IVST)^3-(LVID)^3)x0.8+0.6.$

(LVID: left ventricular internal diameter, IVST: thickness of the intraventricular septum, PWT: posterior wall thickness, 1.04 is the specific gravity of the myocardium, 0.8 is a correction factor. All measurements were performed R synchron at end-diastole and expressed in cms [168].) Left ventricular EF, in absence of regional wall motion disturbances or asynchrony, was approximated by the Teicholz formula [169]. Right ventricular (RV) end-diastolic diameter was measured on the apical 4-chamber view at the middle level [170]. Global systolic function was approximated by measurement of tricuspid annular plane systolic excursion (TAPSE), calculated by the difference between the end-diastolic and end-systolic excursion of the tricuspidal annulus on an M-mode picture (measured in mm) [171]. Specific aspects of right heart assessment are provided in an article by Celermajer et al [172] and Rudski et al [173].

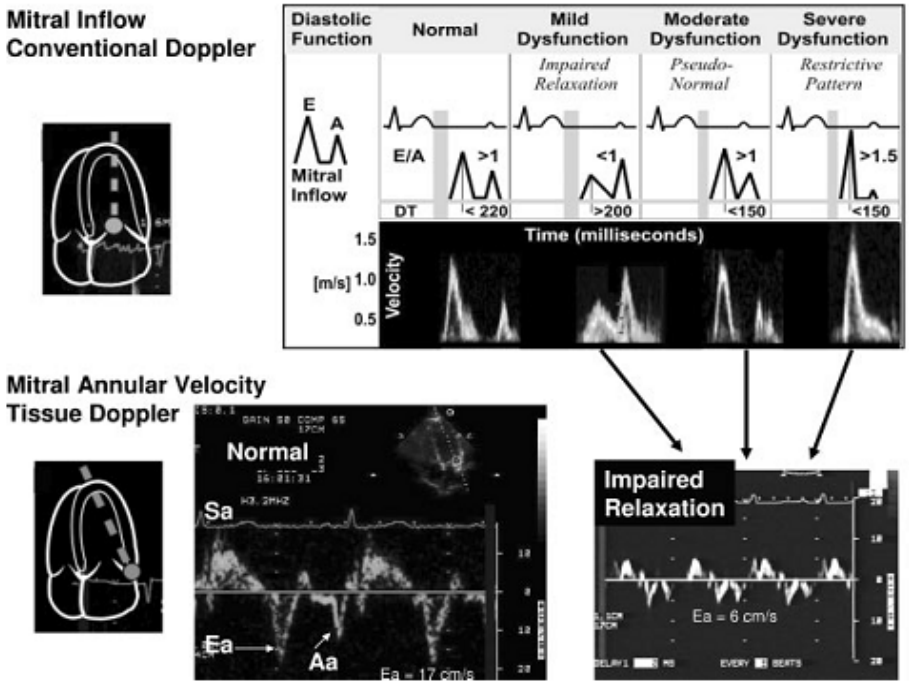

Figure 6. Analysis of mitral inflow and mitral annular velocity by conventional and Tissue Doppler. From: Ho et al.[174]

Conventional Doppler assessment of left ventricular inflow was performed with the pulsed wave Doppler sample volume placed in between the tip of the mitral valve leaflets from the apical 4-chamber view. Global diastolic left ventricular function was determined by peak velocities of E and A wave (m/s) and the E/A ratio, DT (deceleration time) of the E wave (msec) as described by Ho et al [174] , **Figure 6**.

In addition, the sum of the isovolumic relaxation time (IVRT, ms) and isovolumic contraction time (IVCT) were determined as the difference between x-e, where x corresponds to the time interval between the cessation of the mitral inflow and the beginning of the next E wave, and e is the left ventricular ejection time measured from the apical 5-chamber view with the PW sample volume just below the aortic valve [175]. LV TEI index (or mycardial performance index, MPI) was calculated as (x – e)/e. IVRT is defined c-d, where c is the time between the peak of R wave on the ECG to the beginning of mitral E wave and d is the time interval between the R wave and the end of left ventricular outflow. IVCT can be calculated by subracting IVRT from (x-e), see **Figure 7.**

Conventional Doppler RV diastolic indices were determined from the apical 4-chamber view, placing the sample volume between the tips of tricuspid valve leaflets. Global right ventricular filling was characterized by assessment of tricuscpidal inflow E and A peak velocities (m/s), E/A ratio and E wave deceleration time (msec). RV TEI Index was calculated after measurement of the time interval (x) between the end of the tricuspid A wave and the beginning of tricuspid inflow (E) and the duration of the right ventricular ejection wave (e) from the parasternal short-axis view at the level of the aortic valve [176], again with the formula (x-e)/e.

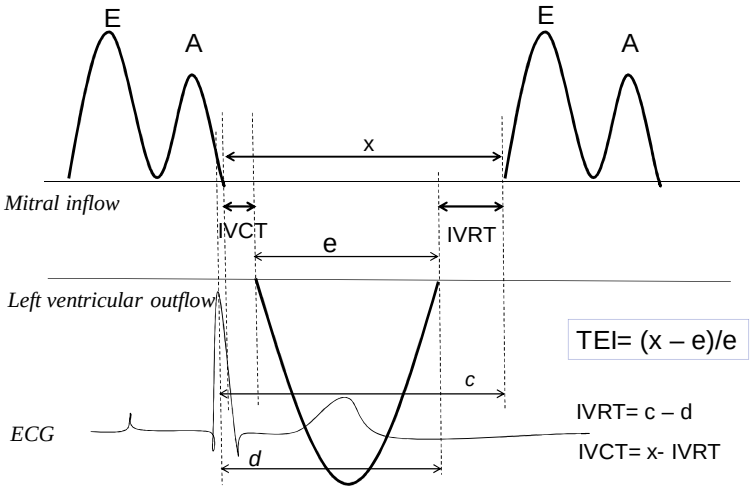

Figure 7. Calculation of myocardial performance index (TEI index). IVRT: isovolumic relaxation time, IVCT: isovolumic contraction time. e: ejection time. For further details see text.

Non-invasive measurement of the pulmonary artery systolic pressure was calculated in all the patients of the study using continuous wave Doppler recordings of tricuspid regurgitation, according to the modified Bernoulli equation. In particular, pulmonary artery systolic pressure was considered as equal to 4 times the square of the peak velocity of the tricuspid jet, plus the right atrial pressure [177]. Inferior vena cava diameters and inspiratory collapse were measured from the subcostal view.

Tissue Doppler myocardial imaging was performed similarly to the technique described by d'Andrea et al [167], by spectral pulsed Doppler signal filters, decreasing aliasing velocities to 15-20 cm/s (close to myocardial velocities), and using minimal optimal gain [178] (**Figure 6)**. Pulsed wave tissue Doppler echocardiography [179] is a valuable tool in non-invasive assessment of myocardial contraction and relaxation as well as fine evaluation of biventricular function by patients with normal EF. Briefly, longitudinal velocities of the left and right ventricles are assessed to identify systolic and diastolic dysfunction [180]. Systolic lateral mitral annular velocity (S_a), and two diastolic velocities early (Ea) and atrial (Aa) as well as tricuspidal annular peak systolic velocities were measured and expressed in cm/s. E/Ea ratio was determined as an index of LV diastolic function [181], values below 8 were considered normal, values above 15 were abnormal, in case of values 8-15, other examination results / parameters were taken into account to determine the presence of diastolic dysfunction.

A peak systolic tricuspid annular velocity less than 11.5 cm/s is related to a RV EF of <45% [182]. This method has also been compared to cardiac MRI [183-186] and has been proved to be appropriate to follow right ventricular function in SSc.

After obtaining a good apical 4-chamber view, a pulsed Doppler sample volume of 5 mm was placed on the RV lateral wall at the base, middle and apex and at the base of the interventricular septum and left ventricular lateral wall, respectively. Apical view was chosen to minimize the angle between longitudinal wall motion and the Doppler beam. Myocardial peak velocity of the systolic wave Sm (m/s), as well as myocardial early (Em) and atrial (Am) peak velocities (m/s) and Em/Am ratio, were measured. Right ventricular free wall peak systolic velocity (the highest of the pulsed tissue Doppler velocities recorded at either the base, middle or apical level) was registered and evaluated for each patient.

4.3.2. ECG monitoring

Resting 12-channel ECG as well as 24-hour 3-channel ECG monitoring was performed according to institutional standard practice. A conventional Holter monitor (Cardiospy, Labtech Kft., Debrecen, Hungary) was fitted by a cardiac technician and returned at 24 hours to the cardiac

investigation laboratory for interpretation. Holter monitor data were analyzed by the same physician investigator (OT), with the help of the Cardiospy software Version 4.03. Arrhythmias categorized as extrasystole, supraventricular tachycardia (>4 beats, without atrial fibrillation or flutter), atrial fibrillation/flutter (>4 beats), pause (>3 seconds was regarded as significant), atrioventricular block (I. degree, Mobitz type I. of II, or third-degree atrioventricular block), ventricular tachycardia (>4 beats), or polymorphic ventricular tachycardia/ventricular fibrillation.

4.3.3. Evaluation of physical condition

The 6-minute walk test was performed prior to rosuvastatin therapy and at end of the treatment period following resting vascular examinations according to ATS Statement Guidelines [187].

4.4. Laboratory analyses

Serum biochemical markers and high sensitivity CRP (hsCRP) were measured using a Modular P-800 analyzer (Roche Ltd, Mannheim, Germany). Serum total cholesterol, triglyceride and uric acid levels were determined by enzymatic colorimetric assay, HDL and LDL-cholesterol were analysed by homogenous enzymatic assay. Serum glucose and urea levels were assessed using enzyme kinetic UV assay, serum creatinine was measured by the compensated Jaffe kinetic method. Estimated GFR was calculated from serum creatinine by the MDRD 175 (Modification of Diet in Renal Disease study group) formula. hsCRP was determined by wide range immunoturbidimetric assay, hsCRP levels> 5 mg/l were considered elevated. Plasma levels of circulating vWF antigen, a marker of endothelial cell activation were measured by STA Liatest vWF immunoturbidimetric assay using microlatex particles coated with polyclonal rabbit anti-human vWF antibodies (Diagnostica Stago, Asnieres, France). After mixing the reagent with plasma, degree of agglutination was evaluated which was proportional to the amount of vWF present in the plasma sample. The reference range for the test is 50-160%. Hematological parameters including hemoglobin (Hgb), white blood cell and platelet counts were determined using an automated hematology analyzer (Sysmex XE-2100D, Sysmex Corp., Kobe, Japan). Erythrocyte sedimentation rate (ESR) was determined by the Westergren method. All the above laboratory measurements were performed by the Department of Laboratory Medicine at the University of Debrecen.

Circulating immune complexes (IC) were detected by the polyethylene glycol precipitation method. Serum complement C3 and C4 levels were measured by nephelometry on a Siemens-Dade-Behring BN-II nephelometer. The applied laboratory reference ranges were as follows: 0.9 to 1.8 g/L

for C3, 0.1- 0.4 g/L for C4 and an extinction of 0-170 for IC. Anti-ENA and anti-Scl-70 autoantibodies were detected by indirect immunofluorescence staining and ELISA technique. Immunolaboratory measurements were all performed by the Regional Immunological Laboratory of the Institute of Medicine at the University of Debrecen.

4.5. Statistical analysis

Results of our cross-sectional as well as longitudinal studies were expressed as the mean±S.D. in case of a normal distribution. Statistical analysis was performed with the help of the SPSS version 11.0 Software, normal distribution was determined by the Kolmogorov-Smirnov test. In case of a normal distribution, statistical analysis was carried out by Student`s paired, one- or two-tailed t-test, according to the individual analysed parameter. Nonparametric distribution was analysed using the Mann-Whitney test. A p value less than 0.05 was considered significant.

Normally distributed parameters were correlated using Pearson`s correlation coefficient, an r value at the $p < 0.05$ level were considered significant. If the distribution of the parameters was not normal, Spearman-test was used to search for correlations. In case of a correlation, the independent variables were plotted in a frame of reference, and the type of correlation was described. In case of a linear correlation, the equation and slope of the function, as well as the regression coefficient (r) value and level of significance (p) were determined.

5. RESULTS

5.1. Endothelial function and ccIMT in SSc

Among the SSc patients undergoing the first study, organ manifestations were as follows. All patients had RP (100%), and 13/29 (45%) had digital ulcers. The second most frequent manifestation was GI (72%) involvement. Pulmonary vascular or interstitial involvement was detected in 19/29 (66%) of patients. Cardiac abnormalities including conduction disturbances, increased right ventricular pressure, diastolic dysfunction as well as left ventricular dysfunction were present in 19/29 patients as well, however, only 10% of patients had renal manifestations. Frequency of anti-topoisomerase I (anti-Scl70) antibody positivity was 44.8 %, patients who belonged mostly to the diffuse cutaneous subgroup, while 10% of all patients were positive for anti-centromere antibody. With regards to therapy, 69% of patients received ACE inhibitors, 59% were on calcium channel blockers and 3/29 (10%) of patients took β-blockers regularly, however, these drugs elicited no significant influence on FMD, ccIMT, or NMD results of the patients compared to drug-naive patients. Parenteral prostacyclin was suspended for a minimum of 6 weeks preceding study measurements. There was no significant difference between patients and controls with respect to age, systolic and diastolic blood pressure values or lipid parameters (**Table 5**). Age and disease duration did not correlate with each other in the studied SSc group (Pearson`s r (27)=0,181, p=0,348.)

FMD in SSc patients was significantly lower, however, NMD was comparable to results of controls (**Table 5**). There was a tendency of higher ccIMT in patients than controls, this, however, remained statistically non-significant (p=0.067). Comparison of the lcSSc and dcSSc form of the disease revealed no differences in FMD, NMD, or ccIMT between the two groups. Neither the studied markers of endothelial function, nor ccIMT corresponded to digital ulcer status of patients (data not shown).

5.2. Relationship of ccIMT, FMD, NMD with age or clinical data in SSc

In search of a connection between markers of endothelial function, subclinical atherosclerosis and age or disease duration, FMD, NMD and ccIMT values were analysed in relationship with age in controls, as well as with age, disease duration, clinical manifestations and autoantibodies in SSc patients.

In the control group, ccIMT showed a significant linear correlation with age (r=0.61; P=0.003) (**Figure 8**), but neither FMD (r=0.264, P=0.082) nor NMD (r=0.032,P=0.870) correlated with age.

Table 5. Comparison of patients and controls in the FMD-NMD-ccIMT study (Study 1)*

	Patients (n=29)	Controls (n=26)	P value
Age (yrs)	51.8±10	49.3±6	n.s.
SBP (mmHg)	136±9	133±13	n.s.
DBP (mmHg)	86±8	85±7	n.s.
TC (mmol/l)	5.39±1.19	5.45±0.9	n.s.
LDL-C (mmol/l)	3.63±1.25	3.13±0.84	n.s.
HDL-C (mmol/l)	1.47±0.4	1.68±0.42	n.s.
TG (mmol/l)	1.24±0.5	1.39±1.0	n.s.
BMI (kg/m^2)	22.8±3.0	24.7±2.7	n.s.
FMD (%)	4.82±3.76	8.86±3.56	p<0.01
NMD (%)	19.13±17.68	13.13±10.40	p=0.129 (n.s.)
ccIMT (mm)	0.67±0.26	0.57±0.09	p=0.067 (n.s.)

*Abbreviations: n.s.: non-significant; SBP: systolic blood pressure; DBP: diastolic blood pressure.

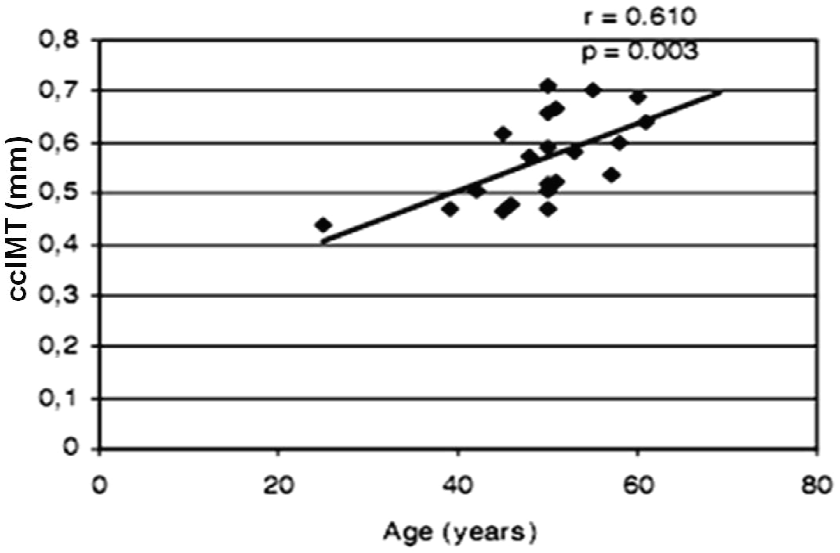

Figure 8. Correlation of ccIMT with age in the control group

ccIMT showed a significant correlation with age in the SSc patients as well (r=0.470; p=0.013), however, in SSc, ccIMT also correlated with disease duration (r=0.472, p=0.011, **Figure 9).**

In contrast to controls, in SSc, an inverse correlation was observed between NMD and age (r=0.492; p=0.012), but no correlation was found between NMD and disease duration. Neither age, nor disease duration correlated with FMD results in SSc (**Table 6**). Interestingly, FMD, NMD and ccIMT did not correlate with each other, nor with the presence or absence of any of the assessed organ manifestations or autoantibody positivity.

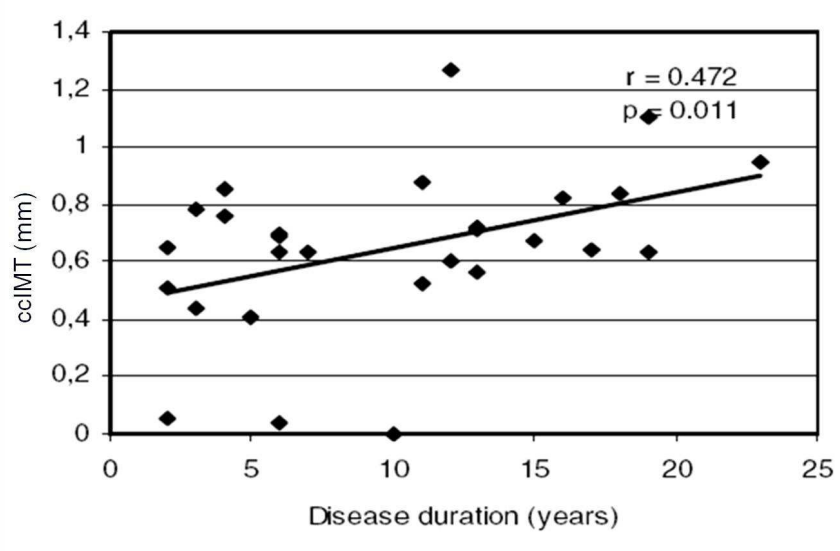

Figure 9. Correlation between ccIMT and disease duration in SSc

Table 6. Correlation of endothelial function and ccIMT with age (and disease duration) in patients and controls (Study 1)

Parameter 1	Parameter 2	R	P	Significance
Controls:				
ccIMT	Age	0.61	**0.003**	+
FMD	Age	-0.264	0.082	-
NMD	Age	0.032	0.87	-
SSc group:				
ccIMT	Age	0.47	**0.013**	+
ccIMT	Disease duration	0.472	**0.011**	+
FMD	Age	-0.364	0.052	-
FMD	Disease duration	0.039	0.842	-
NMD	Age	-0.492	**0.012**	+
NMD	Disease duration	-0.222	0.287	-

5.3. Assessment of arterial stiffness in SSc

Both PWV (9.67± 2.08 m/s vs. 8.00 ± 1.46 m/s, p = 0.00017) and AIx (9.02± 30.32 in SSc vs. –41.15 ± 22.5, p < 0.0001) were substantially higher in SSc patients compared to controls. Upon

comparison of the lcSSc group with the dcSSc patients, PWV of the limited group was significantly elevated (10.04 ± 2.01 m/s vs 8.39 ± 1.87 m/s, respectively; p = 0.034) (**Table 7**).

Differences between lcSSc and dcSSC patients with respect to serum lipid profile as well as disease duration were nonsignificant, however, patients with the limited forms of the disease included in our study were significantly older (mean age of 61.7 vs 45.0 years, respectively.) On the other hand, no statistically significant difference regarding AIx results were observed between patients belonging to different disease subsets.

Table 7. Comparison of SSc subsets with regards to stiffness parameters, main lipid parameters, age and disease duration (Study 2)

Parameter	Diffuse SSc (n=9)	Limited SSc (n=31)	P
AIx (%)	-4.04±36.2	11.75±24.49	n.s. (p=0.296)
PWV (m/s)	8.39±1.87	10.04±2.07	0.034
Total cholesterol (mmol/l)	4.61±1.34	5.24±1.00	n.s.(p=0.136)
Triglyceride (mmol/l)	1.53±0.61	1.4±0.61	n.s. (p=0.558)
Disease duration (yrs)	11.67±7.21	12.74±6.67	n.s. (p=0.678)
Age (yrs)	45.2±8.73	61.74±10.57	0.000125

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5.4. Relationship between AIx, PWV and age or disease duration

A statistically significant, positive correlation was found between AIx and PWV in patients with SSc (R = 0.32, p = 0.045). In addition, AIx, as well as PWV showed significant positive correlations with advancing age in patients with SSc (r = 0.31, p = 0.048 and r = 0.36, p = 0.021, respectively) (**Table 8.**)

Table 8. Correlations between augmentation index (AIx), pulse wave velocity (PWV) and age, serum triglyceride (TG) and total cholesterol (Chol) levels (Study 2)

Variable 1	Variable 2	R	P	Significance
AIx	Age	0.31	0.048	+
PWV	Age	0.36	0.021	+
AIx	PWV	0.32	0.045	+
PWV	TG	-0.995	0.541	n.s.
PWV	Chol	0.0783	0.631	n.s.
AIx	TG	0.0136	0.934	n.s.
AIx	Chol	0.1216	0.455	n.s.

PWV also showed a significant positive correlation with disease duration in SSc patients (r = 0.40, p=0.011) (**Figure 10.)** Disease duration and age did not correlate significantly in this study (Pearson`s R (38)=0,301, p=0,059.) On the contrary, AIx showed no correlation with disease duration (data not shown). We couldn`t detect a relationship between serum lipid levels and either of the assessed arterial stiffness parameters (**Table 8**).

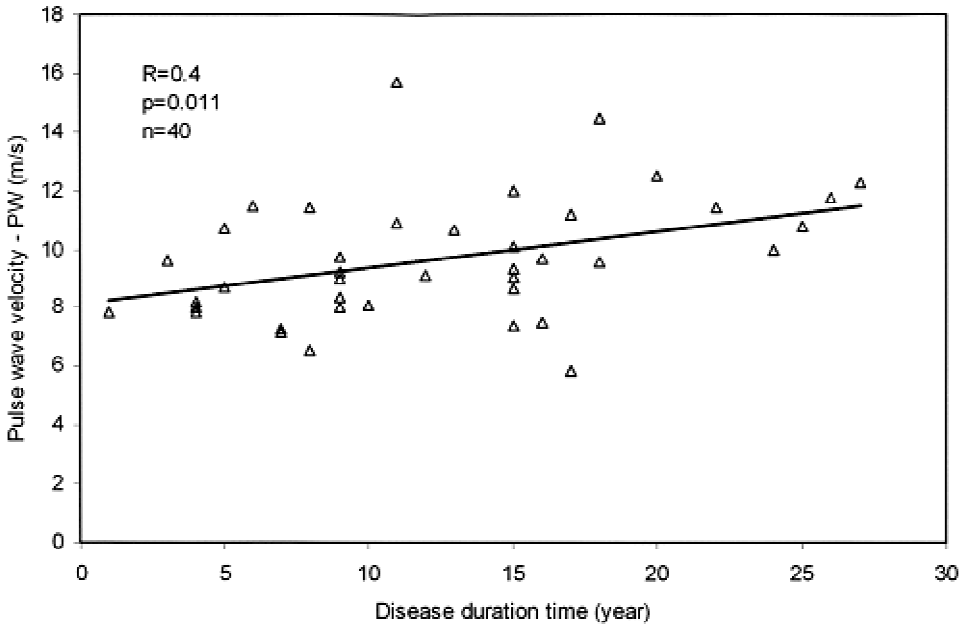

Figure 10. Linear correlation between disease duration and PWV in SSc.

5.5. Effects of rosuvastatin on micro-and macrovascular parameters

Brachial artery FMD significantly improved after six months of rosuvastatin therapy (2.3% ± 3.3% before versus 5.7% ± 3.9% after treatment,P= 0.0002). Altogether 23 patients responded with an increase in occlusion-provoked vasodilation of the brachial artery following rosuvastatin treatment. With regard to patient subsets, FMD significantly improved in the 21 lcSSc patients, from 2.1% to 5.6% (P=0.001). In the seven dcSSc patients, we observed a tendency of improvement in FMD, from 3% to 6% (P= 0.25). The non-significant change in dcSSc may be the result of low patient number (**Table 9**). Upon comparison of FMD results of patients on CCB, and/or BB, and/or nitrates with patients not taking these drugs, we found no significant differences between the two groups. Similarly, immunosuppressive or low-dose steroid therapy did not influence FMD results (data not shown.)

In 11 of the 28 patients (39.3%), baseline c-fPWV values were above the average reference values of age-, lipid- and blood-pressure-status-matched European patients [188]. Although mean

PWV values decreased, neither a-fPWV nor c-fPWV showed a statistically significant improvement upon rosuvastatin treatment (a-f PWV: 8.8 ± 2.2 m/s before versus 8.3 ± 2.1 m/s after therapy, p=0.15; c-f PWV: 8.7 ± 2.6 m/s before versus 8.1 ± 1.9 m/s after treatment, p= 0.1)(**Table 10**). By the end of rosuvastatin treatment, however, only 5/28 patients (17.9%) had c-fPWV above the mentioned reference values.

Table 9. Clinical data of patients with different disease subtypes (Study 3).

Parameter	Limited SSc (n=21) mean (SD)	Diffuse SSc (n=7) mean (SD)	P value
Age	64.4 (8.9)	48.6 (8.4)	**0.0003**
Women (%)	90%	86%	-
BMI (kg/m2)	23.4 (4.2)	22.1 (3.8)	0.455
Disease duration (years)	14.8 (8.0)	9.9 (5.9)	0.144
FMD 1 (%)	2.1 (3.4)	3.0 (3.3)	0.557
FMD 2 (%)	5.6 (3.3)	6.0 (5.7)	0.85
Right ccIMT 1 (mm)	69.5 (15.5)	61.6 (8.8)	0.21
Right ccIMT 2 (mm)	70.4 (15.4)	61.0 (6.6)	0.133
Left ccIMT 1(mm)	73.8 (18.8)	65.3 (9.5)	0.271
Left ccIMT 2(mm)	73.0 (17.7)	61.3 (7.6)	0.104
c-fPWV 1(m/s)	9.2 (2.6)	7.1 (2.1)	0.059
c-fPWV 2(m/s)	8.7 (1.5)	6.2 (1.5)	**0.008**
a-fPWV 1(m/s)	9.2 (2.0)	7.6 (2.4)	0.103
a-fPWV 2 (m/s)	8.9 (2.0)	6.8 (1.9)	**0.018**
CRP 1 (mg/l)	5.0 (5.2)	5.5 (5.6)	0.827
CRP 2 (mg/l)	3.1 (2.1)	4.2 (4.0)	0.343
vWF 1 (%)	228.8 (94.6)	151 (35.6)	**0.046**
vWF 2 (%)	208.4 (80.5)	151 (37.4)	0.083

Table 10. Vascular assessments before and after rosuvastatin treatment in SSc patients (Study 3).

Parameter	Pre-treatment mean (S.D.)	Post-treatment mean (S.D.)	P value*
FMD (%)	2.3 (3.3)	5.7 (3.9)	**0.0002**
Right ccIMT (mm)	0.675 (0.144)	0.681 (0.142)	ns (0.38)
Left ccIMT (mm)	0.717 (0.172)	0.701 (0.165)	ns (0.3)
Number of patients with carotid plaque	5/29 (17.2%)	5/29 (17.2%)	Ns
c-fPWV (m/s)	8.7 (2.6)	8.1 (1.9)	ns (0.1)
a-fPWV (m/s)	8.8 (2.2)	8.3 (2.1)	ns (0.15)
Right ABI	1.1 (0.16) (range:0.6-1.44)	1.1 (0.27) (range:0.2-2)	ns (0.4)
Left ABI	1.1 (0.14)	1.1 (0.19)	ns (0.4)

	(range:0.57-1.38)	(range:0.43-1.6)	
Laser Doppler acceleration slope (U/s)	14.6 (14.8)	10.0 (10.3)	ns (0.08)
Laser Doppler deceleration slope (U/s)	-1.13 (0.92)	-0.64 (1.09)	**0.021**

Mean ABI, as indicator of PAD, was 1.1 ± 0.2 on both sides and remained unchanged after rosuvastatin therapy **(Table 10)**. In one patient, we detected an ABI below 0.6, whose Doppler examination revealed significant stenosis of the right popliteal artery. In 2 additional patients, ABI assessment was not informative regarding atherosclerosis hence it was indicative of incompressible arteries or media sclerosis (ABI values between 1.4-2).

Ultrasound analysis of the common carotid arteries revealed a mean ccIMT of 0.68 ± 0.14 mm on the right and 0.72 ± 0.17 mm on the left side at baseline. Additionally, in 6/28 patients (21.4%), a carotid plaque causing no or nonsignificant stenosis was observed. After rosuvastatin therapy, cc IMT values were 0.68 ± 0.14 mm (P= 0.38) and 0.70 ± 0.17 mm (p=0.3), respectively **(Table 10).** We did not detect additional carotid plaques compared to baseline. Thus, statin treatment did not result in any improvement in carotid atherosclerosis. Only one patient had both lower limb arterial stenosis and manifest carotid atherosclerosis, thus total number of patients with abnormal carotid or ABI findings was 8/28 (28.6%.)

LDPM analysis of the forearm skin flow during PORH testing revealed decreases in the acceleration and deceleration slope of the curves following rosuvastatin therapy compared to pretreatment values (acceleration slope: 14.6 ± 14.8 versus 10.0 ± 10.3 U/second, P= 0.081; deceleration slope: -1.13 ± 0.92 U/second versus -0.64 ± 1.09 U/second, P= 0.021) (**Table 10**). Neither basal, nor peak, or biological zero skin perfusion, nor AH nor any of the reproducible time characteristics (TM, TH1, TH2) showed significant changes compared to pretreatment values (data not shown).

There was no correlation between age and disease duration (Pearson`s R=(26)= 0,111, p=0,573).

5.6. Effect of rosuvastatin on laboratory values in SSc

Presence of antinuclear autoantibodies (ANA) among patients was the following: 26/28 patients (93%) were ANA positive, 12/28 patients (43%) had antibodies against extractable nuclear antigen (ENA), 1/28 (4%) against nuclear ribonucleoproteins (RNP), none against Smith antigen (Sm), 3/28 patients (11%) against SS-A (Ro) antigen, none against SS-B (La). 12/28 patients (43%) tested positive for antibodies against topoisomerase I (Scl-70), and 2/28 (7%) were positive for antibodies against histidyl-tRNA synthetase (Jo-1). Baseline serum lipid levels indicated that 10% of

patients had hypertriglyceridaemia (TG >2.3 mmol/L), 50% had hypercholesterolemia (total cholesterol >5.2 mmol/lL) and 32% had elevated LDL-C levels (>3.4 mmol/L). At baseline, 11 out of 28 patients (39%) had low HDL-C levels (<1.2/<1.0 mmol/L for women/men, respectively). Reference values were determined as recommended for the medium cardiovascular risk group based on the European SCORE chart [30].

Among blood chemistry values, lipid parameters showed significant improvement after six months of rosuvastatin therapy. Mean TG levels decreased from 1.70 ± 0.97 mmol/L to 1.30 ± 0.46 mmol/L following therapy (P= 0.0004). Total cholesterol decreased from 5.3 ± 1.6 mmol/L to 4.2 ± 1.3 mmol/L (P= 0.0003), LDL-C levels decreased from 3.0 ± 1.3 mmol/L to 2.2 ± 1.0 mmol/L (P= 0.0046), while mean HDL-C levels remained unchanged (1.5 ± 0.8 mmol/L before versus 1.5 ± 0.6 mmol/L after therapy, p= 0.33, **Table 11**). Non-HDL cholesterol levels also displayed a significant decrease after statin therapy (3.8 ± 1.5 versus 2.5 ± 1.3 mmol/L, P= 0.0003.) Among acute phase reactants, hsCRP levels showed a significant decrease, from 5.1 ± 5.2 mg/L to 3.4 2.7 mg/L (P= 0.01). ESR, renal function tests and full blood counts showed no biologically relevant changes upon statin therapy as compared to baseline values.

Baseline circulating vWF antigen levels were abnormally high in 63% of patients and although mean vWF antigen levels showed a slight decrease after rosuvastatin treatment (209 ± 90% versus 193 ± 76%), this change remained statistically insignificant (P= 0.09) **(Table 11)**. Serum IC levels were initially elevated and levels returned to normal after rosuvastatin therapy (extinction: 183.6 versus 135.5, respectively, P= 0.007), while C3 (1.81 versus 1.62 g/L) and C4 levels (0.33 versus 0.27 g/L) displayed a significant decrease after rosuvastatin treatment (P= 0.001) within the reference range.

Table 11. Laboratory results of SSc patients before and after the rosuvastatin treatment period (Study 3).

Parameter	Pre-treatment mean (S.D.)	Post-treatment mean (S.D.)	p value
Erythrocyte sedimentation rate (mm/h)	21 (15.6)	24.7 (19)	ns (0.15)
CRP (mg/l)	5.1 (5.2)	3.4 (2.7)	**0.01**
Glucose (mmol/l)	5.3 (1.0)	5.5 (1.3)	ns (0.24)
Urea (mmol/l)	6.0 (2.4)	5.9 (2.5)	ns (0.5)
Creatinine (μmol/l)	67.5 (19.7)	63 (16.9)	ns (0.062)
GFR (ml/min)	82.1 (14.0)	84.8 (10.8)	ns (0.078)
Triglyceride (mmol/l)	1.7 (0.97)	1.3 (0.46)	**0.0004**
Total cholesterol (mmol/l)	5.3 (1.57)	4.2 (1.28)	**0.0003**
LDL (mmol/l)	3.0 (1.3)	2.2 (1.0)	**0.005**
Non-HDL (mmol/l)	3.8 (1.5)	2.5 (1.3)	**0.0003**
HDL (mmol/l)	1.5 (0.84)	1.5 (0.6)	ns (0.33)

Atherogenic index: $\log_{10}$(TG/HDL)	0.08 (0.345)	-0.07(0.22)	**0.0025**
Uric acid (μmol/l)	263 (56)	273 (77)	ns (0.22)
von Willebrand factor (%)	209 (90)	193 (75.6)	ns (0.092)
Haemoglobin (g/l)	125 (12.7)	126 (12.5)	ns (0.242)
White blood cell count (10^9/l)	6.7 (2.6)	7.1 (2.7)	ns (0.191)
Platelet count (10^9/l)	250 (62)	265 (64.5)	ns (0.064)
Complement 3 (g/l)	1.81 (0.4)	1.62 (0.32)	**0.001**
Complement 4 (g/l)	0.31 (0.13)	0.27 (0.1)	**0.001**
Immune complex (extinction)	183.6 (110)	135.5 (55)	**0.005**

With the exception of baseline vWF levels, which were higher in SSc patients with the limited form of the disesase, there were no significant differences in any laboratory parameters before or after rosuvastatin treatment in lcSSc versus dcSSc (**Table 9**).

5.7. Cardiac involvement in SSc and the cardiac effects of rosuvastatin

5.7.1. Echocardiography

Standard echocardiography detected normal left ventricular dimensions along with normal left ventricular systolic function as indicated by the EF (60.5 vs. 61.7%) both before and following 6-month rosuvastatin treatment. Right ventricular diastolic diameter was not increased compared to reference values, and both preceding and following treatment, 25% of patients presented with mild elevations in systolic PA pressure (>37 mm), possibly indicating PAH [189]. Among the other right heart parameters recommended to assess in suspected PAH, (right atrial area>28 cm^2, TAPSE<1.6 cm, pulsed Doppler peak tricuspid annular velocity<10 cm/s raise suspicion of PAH) there was only one data set indicative of PAH, one patient had poor right atrial diameters along with decreased TAPSE, however, peak tricuspid annular velocities, assessed by tissue Doppler, were all normal (data not shown). Detailed results are represented in **Table 12**.

Table 12. Standard echocardiography results before and after rosuvastatin therapy (Study 3).

Parameter	Pretreatment mean ± (S.D.)	Post-treatment mean (±S.D.)	p value
Aortic root (mm)	28.2±2.8	29.1±3.7	0.247
Separation (mm)	18.6±2.9	18.3±2.7	0.428
Left ventricular systolic diameter (mm)	29.9±4.8	28.6±4.3	**0.036**
Left ventricular diastolic diameter (mm)	47.4±5.3	46.8±5.2	0.304
Left ventricular mass index (g/m^2)	63.6±20.1	62.8±21.8	0.76
Intraventricular septum thickness(mm)	10±1.8	10.1±1.5	0.688
Posterior wall thickness (mm)	9.6±1.4	10.15±1.5	**0.050**

Left atrium (mm)	35.4±5.5	36.2±5.5	0.73
Left ventricular ejection fraction (%)	60.5±5.3	61.7±5.2	0.295
Left ventricular E/A	1±0.4	1.02±0.5	0.926
Left ventricular DT (msec)	208±60	200±54	0.656
Right ventricular end-diastolic diameter(mm)	26.7± 4.5	28.4±5.4	**0.023**
Right atrium width (mm)	35.8±6.6	35.7±6.4	0.847
Right atrium length (mm)	44.6±6.6	46±8.1	0.786
Right ventricular pressure (mmHg)	30.6±11.7	29.7±11.4	0.92
TAPSE (mm)	23.3± 4.6	22.6±3.1	0.56
Right ventricular TEI index	0.24±0.19	0.3±0.50	0.54

Severe valvulopathy was infrequent among the assessed SSc patients. Altogether 5/28 patients had mitral valve prolapse, 18/28 patients had mitral insufficiency, the majority of cases being mild to moderate (<Grade II). Only 2 out of 18 patients had grade II mitral regurgitation. Three patients had mild aortic regurgitation, one patient had a nonsignificant aortic stenosis with a mean gradient of 13.5 mmHg and a peak gradient of 25.2 mmHg.

Left ventricular diastolic function was normal in only 21 % of patients at baseline. 61% exhibited impaired relaxation or mild diastolic dysfunction upon interrogation of mitral valve inflow patterns, 10,7% had moderate and 7% had severe diastolic dysfunction (reversible restrictive pattern). Initially, E/Ea was normal in all patients, post-treatment, we detected mildly elevated E/Ea (8-10) in 3/28 patients **(Table 13.**) With the Nagueh-formula their left ventricular filling pressure was calculated to be 14,3; 12,0; and 11,8 Hgmm, respectively, which is not significantly elevated.

Table 13. Diastolic function in SSc before and after rosuvastatin therapy (Study 3), assessed by conventional PW Doppler analysis of mitral inflow and mitral annulus tissue Doppler velocities.

Pattern	Nr. of patients at baseline	Nr. of patients post-treatment
Normal diastolic function (E/A>1, DT< 220 msec), E/Ea<8	6/28	10/28
Impaired relaxation/mild diastolic dysfunction (E/A<1, DT>200 msec)	17/28	15/28
Moderate diastolic dysfunction / pseudonormal pattern (E/A=1-1.5, DT =150-200 msec)	3/28	1/28
Severe diastolic dysfunction or restrictive pattern (E/A >1.5, DT <150 msec)	2/28	2/28

Table 14. Relevant echocardiographic parameters in the follow-up of SSc [180]

Parameter	Normal value	Relevance in SSc
Systolic-diastolic LV wall diameters	35-55 mm	Systolic LV function, enlargement

LV ejection fraction (Simpson)	≥55%	↓indicates systolic LV dysfunction
Left atrial diameter (parasternal view)	28-41 mm	↑ indicates LA enlargement (i.e. mitral insufficiency, etc)
Detailed valvular evaluation		Valvular stenoses and regurgitation
Mitral annulus peak systolic velocity:S_M	>7.5 cm/s (control: 10.3 ±1.4)	Indicator of LV contractility
Lateral annulus early diastolic vel.: E_a	>12cm/s (control:21.2±2.8,)	Indicator of LV diastolic function (Ea<8 cm/s suggests impaired relaxation)
Systolic tricuspidal annular velocity S_T	>11.5 cm/s (control:16.3±0.6)	RV contractility
LV filling pressure, E/E_a	<15 mmHg	Corresponds to E/Ea<10.48
Mean pulmonary arterial pressure (PAP)	<40 mmHg	↑ indicator of RV pressure overload (intersitial lung involvement/ PAH)
TAPSE	>17mm	Lower values signify RV syst. dysfunction [173]

For comparison of values with normal echocardiography results in SSc and to help interpret results see **Table 14**.

Statistically significant changes in the asessed echocardial parameters post-treatment included decreased left ventricular systolic diameter (28.6 vs. 29.9 mm, after and before treatment, respectively, p =0.018), slightly increased posterior wall thickness (10.15 vs. 9.6 mm, p=0.025) and surprisingly, increased right ventricular end-diastolic diameter (28.4 vs. 26.7 mm, p=0.012.)

5.7.2. Arrythmias, conduction disturbances

Results of ECG and Holter ECG monitoring are summarized in **Table 15.** In general, no statistically significant changes were observed comparing ECG-s prior to and post rosuvastatin treatment. Single abnormalities of conduction or ryhthm were infrequent among SSc patients, adding these up resulted in a frequency of conduction disturbances of 23% (6/26 patients), abnormal basic rhythm or major arrhythmias in 42% (11/26) and abnormal HRV (heart-rate variability, indicating some autonomic dysfunction) in 23% (6/26) of patients. Major arrhythmias were defined subjectively as presence of any of the following: supraventricular run or tachycardia, coupled ventricular extrasystole (VES) or ventricular run/tachycardia (VT), a pause longer than 1500 msec or a frequency lower than 60 beats per minute in the active state of the patient. One patient exhibited a prolonged QTc interval (470 msec) after rosuvastatin treatment. This patient also had a 5-beat wide QRS tachycardia at baseline Holter examination, however, Torsade-de-pointes tachycardia or atrial fibrillation could not be detected.

Additionally, we analysed the relationship between all ECG abnormalities (on the resting 12-lead or 24-hour ECG) and laboratory examinations, vascular measurements, echocardiographic findings, as well as disease subtype and presence of pulmonary fibrosis. We categorized patients as

"normal" ECG patients if sinus rythm, normal atrio-ventricular conduction was present without signs of repolarization disturbances on resting 12-lead ECG, and there were no arrhythmias other than isolated, bigemin or trigemin supraventricular or ventricular extrasytoles on the 24 hour ECG monitor. All other patients (those with abnormal basic rythm, conduction distrubances or major arrhythmias, see above) were categorized as abnormal ECG patients.

Our results indicate a strong relationship between physical fitness and normal ECG: patients with a normal ECG performed on average 388±82 m on the baseline 6-minute walk test while among patients with ECG abnormalities this number was 284±103 meters ($p<0.05$), and this difference remained significant after rosuvastatin therapy as well. Not surprisingly, vasoreactivity during the FMD test was smaller in the abnormal ECG-group, however, this difference did not reach statistical significance (3.9 ± 3.1 in the normal group vs. 1.7±3.2% in the abnormal ECG group, $p=0.06$). Patients with abnormal ECG-s had higher pulse-wave velocities (both aorto-femoral and carotid-femoral), corresponding to increased arterial stiffness compared to patients with normal ECG-s, however, only after rosuvastatin treatment did this difference reach statistical significance (c-fPWV was 8.6±1.7 m/s, vs. 6.7±1.6 in the two groups, respectively, $p=0.012$). Presence of a manifest carotid plaque (6/28 patients) stood in no correlation with supraventricular or ventricular arrhythmias, or ST-T segment abnormalities. However, decreased heart rate variability was significantly more frequent in the carotid atherosclerosis patient group ($p=0.044$, OR 8.5, CI: 1.13-63.87.)

Among the assessed laboratory parameters, ESR, CRP and vWF levels or atherogenic indices were in no significant relation with ECG abnormalities of SSc patients. Again, CRP levels of patients with abnormal ECG-s demonstrated a tendency of being higher than CRP-s of normal ECG patients (3.9±2.8 vs. 1.87± 1.28 mg/l, $p=0.059$.)

Table 15. Arrhythmias, conduction disturbances and heart rate variability before and after rosuvastatin treatment (Study 3). The last column ("Total No. of patients"), refers to the total number of patients with the given arrhythmia/abnormality, either before or after therapy. Rosu: rosuvastatin.

ECG characteristics			Before rosu. (n=26) No. of patients	After rosu. (n=24) No. of patients	Total (n=28) No. of patients
CONDUCTION ABNORMALITIES					
		I. AV block	2	1	3
		LBBB	1	1	1
		RBBB	0	1	1
		LAH	0	1	1
		LVH	2	2	2
		RVH	0	1	1
RYTHYM	Basic rhythm	sinus rhythm	24	21	24
		Frequency	72±14	71±11	71.5±12
		atrial fibrillation	2	2	2

	pacemaker rhythm	0	1	1
Minor arrhythmias	SVES (lone/bigeminy)	18	15	25
	VES (lone or bigeminy / trigeminy)	9	8	15
Major arrhythmias	supraventricular run/tachycardia	7	4	11
	ventricular (coupled ES or VT)	4	3	8
	bradycardia <60/min± pause>1500 msec.	5	2	5
HEART RATE VARIABILITY	Normal	20	17	17
	Decreased	5	5	8
	Increased	1	0	1
	non- evaluable	0	2	2
ST-T ABNORMALITIES		3	3	4

Assessment of the echocardiographic findings parallel to ECG results led to the following observations. Left atrial longitudinal dimensions on apical 4-chamber views were significantly higher in patients with abnormal ECG-s (47.9±7.6 vs. 38.3±2.9 mm, p=0.02) In addition, left ventricular DT (deceleration time of the E wave of mitral inflow) was somewhat prolonged in the abnormal ECG-patient group although it did not reach statistical significance (p=0.07). This might indicate left ventricular diastolic dysfunction as one possible cause of the arrhythmias. Among echocardiographic parameters informing about right ventricular systolic function, TAPSE did not differ significantly between the abnormal and normal ECG patient groups (22.6 ± 4.3 mm vs 24.9 ±4.8 mm, respectively, p=0.11), however, right ventricular free wall systolic peak velocity (height of the S wave), assessed by pulsed wave tissue Doppler was significantly higher in patients with abnormal ECG-s (19.6±5.1 vs. 15.4 ± 3.9cm/sec, p= 0.029). We assessed the possibility of higher grade tricuspidal insufficiency explaining these results, and found that the grade of TI did not correlate with the size of the right ventricular maximal S wave with tissue Doppler (Spearman's R=-0.076, p=0.71). With respect to right ventricular diastolic function, right ventricular E wave (55 vs. 65 cm/s) and E/A ratio (1.0 vs. 1.28) were both significantly lower in the abnormal ECG SSc group (p=0.034 0.039 respectively), indicating right ventricular diastolic dysfunction as an additional factor possibly influencing ECG-detectable functional cardiac abnormalities in SSc. Heart rate was indifferent between the two groups, excluding the possibility of bradycardia influencing our results. Longitudinal left atrial diameters, thus left atrial size based on the apical 4-chamber view were significantly increased in the abnormal ECG group, whereas we did not observe any differences in left ventricular filling pressure indicated by the E/Ea ratio. For detailed data and comparison of echocardiac and ECG parameters see **Table 16.**

Table 16. Relationship between baseline echocardiographic characteristics and ECG abnormalities in SSc. (Study 3). Only statistically significant data are shown.

Echo parameter (unit)	Normal ECG group, mean	S.D.	Abnormal ECG group, mean	S.D.	P
Right ventricular e (msec)	348.5	50.7	300.4	41.8	**0.008**
Tricuspid inflow E wave (cm/s)	65.1	8.4	55.2	13.5	**0.034**
Right ventricular E/A	1.28	0.2	1.0	0.3	**0.039**
Left atrial diameter B (mm)	38.3	2.9	47.9	7.6	**0.020**
Left atrial size (AxB, mm2)	1446	172.8	1914.0	531.1	**0.093**
Right ventricular free wall vmax (cm/s)	15.4	3.9	19.6	5.0	**0.029**

5.7.3. Correlations between ECG abnormalites and results of echocardiography

Apart from the comparison of normal and abnormal ECG patient groups we searched for a relationship between echocardiographic findings (especially right ventricular functional parameters) and supraventricular arrhythmias (SVARY), ventricular arrhythmias (VARY), presence of decreased heart rate variability and atrial fibrillation (AF) as well as ST-T-wave abnormalities using Fisher's exact test.

Presence of supraventricular arrhythmias correlated inversely with TAPSE (24.68±4.4 vs. 21.02 ±3.6mm, p=0.031), tricuspid E wave, and right ventricular E/A ratio (1.23±0.27 vs. 0.89±0.29, p=0.004) and a positive correlation was found between SVARY and left atrial enlargement (longitudinal LA diameter 41.5±4.5 vs. 52±10.5 mm).

Ventricular arrhythmias showed a positive correlation with estimated systolic right ventricular pressure (27.1±9.6 vs. 38.6±14 mmHg, p=0.019), mitral inflow E wave deceleration time (DT, 192.2±49.1 vs. 247±69.3 p=0.027) and thickness of the interventricular septum (9.6±1.5 vs. 11.1±2 mm, p=0.04.) Statistically, mitral E/Ea ratio also correlated positively with the presence of ventricular arrhyhthmias, however, this remained clinically insignificant, because E/Ea was normal in all patients.

Decreased HRV correlated inversely with right ventricular e and x times (x=398±52 vs.347 ±65 msec, p=0.04; e=331±42 vs. 279 ±48 msec, p=0.009) as well as tricuspid E/A ratio (1.16 ±0.29 vs. 0.9±0.33 p= 0.049) and posterior wall thickness (9.2±1.2 vs. 10.5 ±1.6 mm, p=0.036). Left ventricular diastolic dimensions were smaller in SSc patients with decreased HRV on the Holter electrocardiogram (48.6 ±4.5 vs. 44.2 ±6.2 mm, p=0.05).

We found a positive correlation between presence of pulmonary fibrosis on CT scan and left atrial enlargement (LA longitudinal diameter 40.8±4.5 vs. 50.5±9.1 mm, p=0.04). Also, a strong inverse correlation between TAPSE and pulmonary fibrosis (25.9±4.35 vs. 21.2 ±3.42 mm, p=0.003)

suggested more frequent right ventricular systolic dysfunction in SSc patients with pulmonary fibrosis.

According to our results, ECG abnormalities were independent from SSc disease subtype (limited-diffuse form).

Case numbers of atrial fibrillation and ST-T segment abnormalities were too small for statistical comparison.

5.7.4. Results of exercise testing

Based on our results, rosuvastatin did not influence physical fitness, as assessed by 6-minute walk test in SSc patients. 6-minute walk-test results prior to and following rosuvastatin treatment were 311±108 vs. 316±118 meters, respectively (p=0.11). It is noteworthy that the typical causes for stopping or pausing the examination beside tiredness were frequently musculoskeletal complaints, rather than complaints about shortness of breath (which was infrequent in this patient group). This may suggest the need for use of exercise tests other than the 6-minute walk test in SSc (such as exercise echocardiography or exercise lung-function tests) [190].

6. DISCUSSION

In a significant proportion of autoimmune rheumatic diseases [43, 191] including RA [192-194], SLE [195-197] or polymyositis [198-200] accelerated atherosclerosis and early cardiovascular disease (presenting as early onset endothelial dysfunction, subclinical atherosclerosis, etc.) are major influencing factors of morbidity and mortality.

6.1. Endothelial function and ccIMT in SSc

At the time of conducting our study of FMD and ccIMT in SSc in 2007, there was little information about endothelial dysfunction in SSc [64, 65, 67] despite increasing number of reports on SSc-related vascular involvement [89, 96, 201]. Our results of decreased brachial FMD in a Framingham–risk matched study group indicate early functional impairment of the endothelium typical of SSc preceding overt carotid atherosclerosis. Simultaneously, D`Andrea et al [65] reported about decreased middle left ventricular strain rate and brachial FMD as well as decreased transthoracic coronary flow reserve in SSc patients, suggesting a clinical relevance of decreased brachial artery FMD going beyond local vasomotor function, indicating definite myocardial and vascular impairment in SSc. FMD in the general population is lower in ischaemic heart disease (IHD) patients compared to non-IHD patients [202] and decreased FMD (lower than 11.3%, or in other studies, <6%) has been found to be an independent predictor of future adverse cardiovascular events in otherwise healthy individuals [203, 204]. A recent metaanalysis of vascular involvement in SSc included only two studies reporting about decreased NMD, one with a case number of 12 encumbering statistical evaluation [68], and one where patients with RP were included together with SSc patients [71]. All other reports supported our findings conveying evidence of decreased FMD and unchanged NMD in SSc compared to controls[16].

Preserved NMD in SSc can be explained by multiple factors. The pathogenesis of SSc involves elevated levels of NO synthase inhibitors, such as ADMA [113, 114], hence decreased bioavaliability of the vasodilator nitric oxyde. The clinical significance of impaired endothelium-dependent vasodilation parallel to maintained nitroglycerine-dependent vasoreactivity is on one hand a possible therapeutic option for introduction of exogenous NO-donors and nitroglycerine in SSc therapy to reduce SSc patients' vascular symptoms and perhaps cardiovascular risk. On the other hand, preserved endothelium independent vasodilation is a major argument supporting the dominant role of regulating vasoactive molecules as opposed to structural vascular alterations (vessel wall

abnormalities, obstructive vasculopathy) in the pathomechanism of SSc, otherwise vasodilation would be impaired even in presence of exogenous vasoactive agents.

The inverse correlation of NMD and positive correlation of ccIMT with age might indicate a deteriorating endothelium-independent vasodilation and advancing atherosclerosis with higher age in SSc. The correlation of ccIMT with disease duration could in theory be due to more advanced age, since age and disease duration may also be related to each other. In this patient cohort, however, age and disease duration were independent, suggesting that ccIMT is somehow related to systemic sclerosis itself.

6.2. Arterial stiffness in SSc

Increased arterial stiffness has been described in SLE and RA and has been reported to correlate with disease duration in these disorders [205, 206]. Elevated aortic PWV has been found to be the best indicator of increased cardiovascular risk independently from traditional risk factors in a post hoc analysis of the Framingham heart study [207]. Thus, in the past decade, arterial stiffness has been investigated to shed light upon the presence and extent of increased cardiovascular risk in systemic sclerosis as well. Our group has been among the first groups to assess indicators of systemic arterial stiffness in SSc ([66, 70, 76]) and was the first to report about elevated pulse wave velocity in SSc compared to healthy controls signifying large-vessel involvement in addition to microvascular disease in SSc. In addition, we also demonstrated a correlation between PWV and SSc disease duration and detected that higher PWV values characterize the limited form of the disease compared to the diffuse subtype, suggesting more severe macrovascular involvement in the lcSSc subgroup. Since publication of our results in 2008, numerous reports supported our findings among others in Italian [208-210], Lithuanian [70], and Chinese [78] SSc patients. Some studies found no significant change in PWV compared to controls [38, 66], but even in the latter study, AIx was found abnormal in SSc. Others measured indicators other than PWV such as fingertip-derived AIx [75]. In one report, elevated PWV has even been found to predict occurrence of new digital ulcers [210] in SSc. Although some authors imply Arteriograph results may better correspond to brachial artery stiffness than to central arterial stiffness [211], invasive validation of the instrument against cardiac catheterization conveyed reassuring correlation of results for clinical use [157].

6.3. Cardiovascular effects of rosuvastatin in SSc

The detected significant improvement in endothelial function assessed following six months of rosuvastatin treatment is a novel finding which has not been demonstrated in SSc patients before. Comparing this effect of rosuvastatin to the vascular effects of atorvastatin treatment reported by other investigators [140, 145, 212], the two statins seem similar regarding their effect on endothelial function (FMD). Whether favourable effects of rosuvastatin on endothelial function are also accompanied by improvement in clinical vascular symptoms such as Raynaud's phenomenon, digital ulceration or Rodnan skin score in SSc has yet to be investigated.

The duration of therapy is a decisive feature of successive statin treatment in SSc. While in one study [140], eight-week atorvastatin (20 mg) treatment in SSc exhibited no effect on endothelial function as assessed by Laser Doppler imaging, another [145] described beneficial effects of 24-month atorvastatin (10 mg) therapy on Raynaud's phenomenon of SSc patients. Thus, positive effects of statins on the vasculature, as well as on the underlying inflammatory disease may most likely be expected only after six months or more. We must also point out that each statin has different structure and lipophilicity (e.g. rosuvastatin is more hydrophilic than atorvastatin), thus their transport through the lipophilic membrane to exert intracellular effects is also different. This means non-lipid-related drug effects may also differ not only according to binding differences but also because one substance reaches the intracellular space in a smaller concentration than the other [213].

By assessing ccIMT, PWV, FMD and ABI we wished to add further data to the debate [37] about the extent and presence of atherosclerosis in patients with SSc. In the present study, mean pretreatment values of right and left ccIMT were within the 25th and 75th percentile range of the given age group as described in largescale European cohort studies [214, 215]. Our current findings support the results of studies reporting normal ccIMT in scleroderma patients (6 studies, total number of 237 patients) and it seems these studies outnumber those detecting abnormally high ccIMT values in SSc [216] (4 studies, 144 SSc patients between 1998-2011). However, during our carotid ultrasound examinations asymptomatic plaques were discovered in almost one fourth of patients as a sign of carotid atherosclerosis despite increased cc IMT.

Abnormal ABI has recently been described in SLE [196] and RA but also Sjögren's syndrome. In contrast, we found that ABI values in SSc tend to be normal, with the exception of individual cases, predominantly in patients with the limited form of the disease. Due to the low number of abnormal ABI results, we could not identify any factors which could help detect increased

risk for abnormal ABI (peripheral arterial disease) in SSc. Our results with roughly one sixth of patients exhibiting mildly decreased ABI are in concordance with previous studies reporting normal [93, 94] or slightly abnormal ABI values in SSc [95, 96]. According to TASC force guidelines [217], ABI values between 0.9-0.99 are borderline and only values lower than 0.9 are to be considered abnormal. Values below 0.4 suggest critical peripheral arterial disease. Bartoli and Kaloudi [93, 94], however, did not report about ABI values below the 0.4 threshold, thus rendering identification of significant peripheral arterial disease difficult. Taking in account newly identified carotid plaques, 8/28 patients (28%) had either an abnormal carotid result or abnormal ABI in our present study, underlining the need for screening of these abnormalities despite normal mean values in SSc.

Although the frequency of PWV results above mean European reference values [188] decreased from 11/28 to 5/28 following rosuvastatin therapy, somewhat suprisingly, baseline a-f and c-fPWV were elevated in less than half of all patients in this study and mean values exhibited no statistically significant changes following rosuvastatin treatment. Multiple explanations, including concomittant use of calcium channel blockers and/or ACE-inhibitors **(Table 4)**, and the duration of statin therapy may be put forward. Also, regional differences in arterial stiffness, thus increased stiffness of the forearm arteries, as suggested by Liu et al [78], is possible explaining why our Arteriograph-derived previous PWV results over the brachial artery may have been greater than ultrasound-measured central aorto-femoral and carotid-femoral PWV. It is always a question wether a patient cohort of longer disease duration responds well to a possibly disease-modifying treatment, thus we cannot declare that rosuvastatin/statin therapy is ineffective in reducing PWV in SSc patients with a newly diagnosed disease as well. In order to interpret our results appropriately, repetition of these examinations with longer treatment periods, perhaps shorter disease duration and comparison with PWV progression of nontreated patients will be required.

We decided to add Laser Doppler measurements to our study protocol because some studies had previously indicated impaired cutaneous vasodilatory response to ischemia in SSc patients [218]. Our Laser Doppler flowmetry measurements revealed significantly slower deceleration slope during PORH testing of the forearm skin after rosuvastatin compared to pretreatment values. However, the relevance of these slopes in determining microcirculation has not yet been fully determined.

Our results on cardiac involvement in SSc suggest several consequences. Whereas all patients had preserved left ventricular systolic function, LV diastolic function was normal in merely 20 % of SSc patients at baseline as a sign of cardiac involvement in as many as 80% of patients. 61% had impaired relaxation upon interrogation of mitral valve inflow patterns, 10.7% had moderate and 7% had severe diastolic dysfunction. Repeated echocardiography pre-and posttreatment demonstrated that rosuvastatin left the most important left ventricular systolic, diastolic and right ventricular

functional parameters (EF, TAPSE, mitral inflow patterns) as well as ECG findings unchanged. We observed a minor decrease in left ventricular systolic diameter, minor increase in posterior wall thickness and increased right ventricular end-diastolic diameter after rosuvastatin therapy, although all of these changes remained within the reference range and their clinical significance, taking in to account unchanged functional parameters, is limited. As one would expect, pulmonary fibrosis on the CT was associated with right ventricular systolic dysfunction indicated by decreased TAPSE, but also correlated with left atrial enlargement.

Our coarse findings of major arrhythmias in 42%, conduction disturbances in 23%, and abnormal heart-rate variability in 23% of SSc patients are in concordance with the frequency of abnormal ECG-s found during retrospective analysis of 256 SSc patients by Draeger et al [219]. Supraventricular arrhythmias were more frequent in presence of right ventricular systolic or diastolic dysfunction (decreased TAPSE or tricuspid E/A ratio), or left atrial enlargement, whereas ventricular arrhythmias correlated with estimated systolic right ventricular pressure, mitral inflow E wave DT and thickness of the interventricular septum in our study. Pulmonary fibrosis also correlated with markers of RV dysfunction (decreased TAPSE and left atrial enlargement in SSc. The mechanism linking these abnormalities is probably the right ventricular overload caused by increased pulmonary resistance resulting in RV systolic and diastolic overload.

Noteworthy is the tendency of lower FMD values and elevated PWV values, and higher CRP values, possible signs of increased vascular involvement we observed in SSc patients with abnormal ECG-s compared to normal ECG patients. However, this reached statistical significance only in case of carotid-femoral PWV after rosuvastatin treatment. Additional characteristics of the abnormal ECG group included greater left atrial dimensions and right ventricular diastolic dysfunction (indicated by both E wave height and decreased E/A ratio). Others [219] also reported that abnormal ECG findings indicated more severe cardiopulmonary involvement. The significant correlation of decreased heart rate variability with carotid atherosclerosis is also a noteable finding in view of the reports of low HRV as a predictor of death in non-SSc patients with heart failure [220]. The correlation between carotid atherosclerosis and decreased heart rate variability might be related to the atherosclerosis involving the artery of carotid body chemoreceptors influencing the sensory afferent function of the heart’s autonomic reflexes. Decreased HRV also correlated with right ventricular diastolic dysfunction assessed by tricuspid E/A ratio and posterior wall thickness. The relationship we detected between physical fitness assessed by 6-minute walk-test and normal ECG is a novel finding: patients with a normal ECG performed 36% better and this difference remained significant after rosuvastatin therapy as well.

Among laboratory parameters, in our current study marked reductions in total TG, total- and non-HDL cholesterol as well as LDL-C cholesterol were observed after six months of rosuvastatin treatment. HDL-C levels remained unchanged after therapy. The changes in total cholesterol and LDL-levels correspond to expectations based on results of clinical trials conducted previously to assess efficacy of rosuvastatin in comparison with other statins [221-223]. A possible explanation for not observing significant changes in HDL-C levels may be the fact that the majority of SSc patients had HDL-C levels within the normal range at baseline.

Among inflammatory markers, hsCRP levels decreased significantly following rosuvastatin treatment. Rosuvastatin reduces CRP levels in low cardiovascular risk individuals in the general population [224].There is substantial evidence that hsCRP is an independent cardiovascular risk factor in the general population, but its predictive value in early stages of atherosclerosis is doubtful [148]. To the best of our knowledge, this is the first study to show that CRP levels improve after six months of 20 mg rosuvastatin therapy in patients with SSc. A Canadian research group [117] recently reviewed 1043 SSc patients (mean ± SD age 55.4 ± 12.1 years, mean ± SD disease duration of 11.0 ± 9.5 years) with respect to CRP levels, limited or diffuse disease subtype, disease activity, disease severity and survival. The scientists reported a mean CRP of 11.98 ± 25.41 mg/l in dcSSc, higher than in lcSSc (8.15 ± 16.09 mg/l.) In our study there were no differences in CRP levels according to disease subsets, and CRP was slightly above reference values in both patient groups. Although not present in all subgroups, authors demonstrated an association between elevated CRP and disease activity (mRSS, HAQ), decreased total lung capacity (<80%), elevated serum creatinine and decreased survival ($p<0.01$). (Elevated CRP levels were defined as >8 mg/l and were found in 25.7% of all patients in the Canadian study.)

Levels of circulating vWF antigen have been found elevated in patients with Raynaud's phenomenon and SSc [225, 226] as a sign of endothelial injury. In our patient cohort, rosuvastatin treatment resulted in a slight, non-significant decrease in elevated circulating vWF levels.

Although the exact pathomechanism of SSc is yet unknown, lately, activation of the complement system has been suggested and immune complex deposition, particularly in the perivascular and subendothelial region, has been described. In our current study, we observed elevated serum immune complex levels returning to normal following rosuvastatin treatment. In addition, C3 and C4 levels showed a significant decrease after rosuvastatin treatment. Notably, according to a recent review on endothelial function in autoimmune rheumatica, high levels of C3, C4 and C5a are associated with an increased risk for the onset of acute events in SSc, and activated complement is more abundant in unstable as compared with stable atherosclerotic lesions, underlining the clinical significance of the C3-, C4- decrease we observed.

In the general population, clinical data about rosuvastatin has been conveyed from multiple studies addressing among others common carotid intima-media thickness (ccIMT) changes during rosuvastatin treatment (METEOR trial: two-year rosuvastatin 40 mg vs. placebo effecting annual ccIMT increase in middle-aged patients with modest ccIMT thickening and elevated LDL), the effect of 10 mg rosuvastatin vs. placebo for 32.8 months in an elderly patient cohort with heart failure on cardiovascular death, nonfatal AMI and stroke (Controlled Rosuvastatin Multinational Trial in Heart Failure: CORONA) or the effect of rosuvastatin on coronary atheromas (A Study to Evaluate the Effect of Rosuvastatin on Intravascular Ultrasound-Derived Coronary Atheroma Burden: ASTEROID study) which offered insight into the effects of rosuvastatin on the atherosclerotic process. Results of these studies showed that 40 mg/die rosuvastatin did not invert carotid atherosclerosis but slowed the rate of ccIMT increase significantly (METEOR) over two years, 10 mg/day rosuvastatin reached a 13% decrease in relative risk of primary and secondary outcomes in heart failure patients with a CRP≥2 mg/l (CORONA) and 40 mg/day rosuvastatin decreased the coronary atheroma burden significantly over 2 years, as assessed by IVUS, and also improved minimum lumen diameters (examined by quantitative coronary angiography, ASTEROID). On the other hand, we could not detect significant effect of 20 mg/die rosuvastatin on cc IMT reduction after 6 months of treatment in SSc. Lower doses of rosuvastatin or perhaps the relatively long SSc disease duration are possible explanations for this fact. A study of primary preventive effects of a 20 mg daily dose of rosuvastatin in patients with normal LDL but moderately increased CRP (>2mg/l, on average 4.6 mg/l) revealed a significant cardiovascular risk reduction within 1.9 years regarding primary MI, stroke, unstable angina, angiological revascularisation or cardiovascular death (JUPITER=Justification for Use of statins in Prevention: an Intervention Trial Evaluating Rosuvastatin trial [148, 150]). In spite of undeniable positive effects of rosuvastatin in the above setting, results and limitations of the JUPITER study are still debated today by many [227-229], due to multiple reasons. Firstly, JUPITER has been terminated early, which in general tends to exaggarate possible benefits in clinical trials. Secondly, lack of conventional risk-reducing therapy on both arms (such as acetylsalicylic acid, weight loss or smoking cessatio) might have influenced results significantly, since these in themselves might have reached a significant reduction of cardiovascular endpoints in the assessed patient cohort. To assess possible cardiovascular risk reduction by rosuvastatin in SSc, we plan to continue patient follow-up for the events also studied in the JUPITER trial for 10 years.

The exact explanation of the beneficial effects observed by rosuvastatin therapy in our SSc patients is not fully elucidated. First question was if endothelial function improvement is linked to a decrease in atherogen lipid levels or is it an independent vascular effect of rosuvastatin? In order to

answer this question, we calculated pre- and posttreatment atherogenic indices for each patient and compared them with FMD values. Although the decrease in atherogenic index after rosuvastatin therapy was statistically significant, no correlation was found between initial or posttreatment FMD values and the atherogenic indices, supporting the notion that the improvement of endothelial function is independent from rosuvastatin`s lipid-lowering effect. The same conclusion was drawn by van Doornum et al [230] who demonstrated that atorvastatin reduced arterial stiffness by 14% in RA. Interestingly, the greatest improvement was observed by van Doornum in patients with the greatest disease activity independently from baseline cholesterol levels.

Rosuvastatin might enhance adiponectin release in humans, as reported by Zhang et al [231], who found a 67% elevation in adiponectin levels compared to controls after rosuvastatin therapy in an animal model. As discussed in the pathophysiology section, adiponectin takes part in conveying antiproliferative effects of peroxisome proliferator-activated receptor gamma (PPAR-γ) on arterial smooth muscle cells. Elevated adiponectin correlates inversely with skin fibrosis and also plays a part in scleroderma-related lung disease [119].

In a study designed to search for markers and signs of endothelial dysfunction and subclinical atherosclerosis among SSc patients, asymmetric dimethylarginine (ADMA) levels in patients with diffuse cutaneous SSc were reported to be significantly elevated compared to healthy controls [113]. 5 mg/kg rosuvastatin therapy in rats has been reported to reduce monocrotaline-induced pulmonary vascular remodeling, right ventricular hypertrophy and dysfunction. In this murine model, rosuvastatin normalized right ventricular dysfunction and decreased ADMA levels [232]. Thus, another possible hypothesis for mechanism of action of rosuvastatin in SSc is normalization of down-regulated pulmonary Akt/p-Akt and eNOS/p-eNOS expressions, and increasing 2 expression parallel to decreasing serum levels of ADMA. Two limitations of the above study could be, however, that the exact degree of concordance between the rat model and SSc in humans is unknown, furthermore, significantly (20x) higher doses of rosuvastatin were applied (5 mg/kg in the animal model vs. ~ 0.2 mg/kg in our study.)

Statins also exert an inhibitory effect on the Rho kinase pathway. This enzyme has an important role linking oxidative stress provoked by cold stimulus to α2-adrenoreceptor activation and translocation to the cell membrane in cutaneous vessels of SSc, a hallmark of vascular tone dysregulation observed in the disease. This pathway also promotes fibroblast-myofibroblast differentiation, extracellular matrix remodelling and tissue fibrosis, no wonder Rho kinase pathway inhibitors have been recommended as therapeutic targets in SSc as early as 2008 [18].

Yet another possibility explaining the mechanism of action of rosuvastatin in SSc is based on the findings of Kwak et al, [132] who demonstrated that statins directly inhibit inducible MHC-II-

expression by IFN-gamma, thus repress MHC-II-mediated T-cell activation, in multiple human cell types including endothelial cells and monocyte-macrophages. As discussed in the introduction at least IL-13 producing Th2-type T-cells are sure contributors to disease pathogenesis in SSc. However, in the current study on rosuvastatin in SSc we have not assessed helper T-cell activation and cytokine production, hence we cannot decide if and to which extent altered T-cell function might have contributed to the detected clinical effects of rosuvastatin.

In conclusion, we highlight the result of the rosuvastatin-SSc study that beneficial rosuvastatin effects are non-lipid related. This is a clinically important finding, since rosuvastatin is currently the most potent lipid-lowering statin, but due to its limited lipophilicity, it is not the best with respect to transmission through the cell membrane, suggesting that if beneficial vascular effects of rosuvastatin are to be reached via non-lipid mechanisms in SSc, atorvastatin may be a much better choice for statin treatment in patients with SSc. The same conclusion was drawn regarding cardiovascular protection in the general population by a comparative review of atorvastatin and rosuvastatin by Di Nicolantonio et al [233].

With respect to cardiovascular risk and the potential role of statins as preventive therapeutic strategies in SSc, we must aggree with Hollan et al [43], authors of a comprehensive review of CV risk in autoimmune rheumatic diseases. It seems clear that patients with autoimmune rheumatic disease in general are undertreated respecting cardiovascular risk. While we are waiting for results of placebo-controlled randomized clinical trials, novel therapeutic options and renewed guidelines for patients with various autoimmune disorders including SSc, it is crucial to at least ensure cardiovascular protection on basis of patients' estimated general cardiovascular risk disregarding their autoimmune rheumatic disease, rather than applying no treatment at all. Improvement of endothelial function and antiinflammatory effects of statins detected in our study as well are definitely desirable and beneficial in SSc. Hopefully, in the near future, increasing evidence on both safety issues and efficacy of statin therapy in SSc will pave the way for renewed guidelines including recommendations of statin therapy for cardiovascular risk reduction for the benefit of all patients suffering from SSc today.

7. NOVEL RESULTS

During the assessment of cardiovascular abnormalities and the effects of rosuvastatin treatment in SSc we got the following novel results:

1. FMD is significantly lower in Hungarian SSc patients compared to controls.

2. FMD, NMD or ccIMT does not correlate with internal organ involvement or autoantibody positivity in SSc.

3. PWV and AIx is significantly elevated signifying increased arterial stiffness in Hungarian patients with SSc.

4. PWV correlates with disease duration in SSc and is significantly higher in limited SSc compared to the diffuse form of the disease.

5. Rosuvastatin improves FMD, lowers serum high-sensitivity CRP and complement levels and decreases immune complex production in patients with SSc indicating an effect on disease activity and on cardiovascular risk.

6. The improvement of endothelial function in SSc, taking into account lack of correlation between FMD and atherogenic index, is due to a mechanism of action other than the lipid-lowering effect of rosuvastatatin.

7. 6-month rosuvastatin therapy has no effect on peripherial arterial disease frequency screened by ankle-brachial index, on ccIMT, on the frequency of carotid plaques or on forearm cutaneous blood flow as assessed by Laser Doppler perfusion imaging.

8. SUMMARY

Systemic sclerosis has one of the worst prognosis among connective tissue diseases. Disease course is determined by internal organ manifestations, disease subtype, as well as concomittant non-SSc related disorders. Cardio-vascular causes account for at least 26% of mortality in SSc nowadays, increasing parallel to improved organ involvement treatment options. Thus, revealing cardiovascular abnormalities is of increasing significance during SSc patient care.

During our studies, we initially assessed endothelial function (flow-mediated dilation, FMD), carotid intima-media thickness (ccIMT, indicator of early atherosclerosis) and parameters of arterial stiffness (pulse wave velocity, PWV, and augmentation index, AIx) in two cross-sectional, controlled studies in two different SSc patient groups. We detected that FMD is significantly decreased indicating an early functional abnormality of the endothelium, while nitrate mediated dilation (NMD) is preserved compared to the control group, supporting therapeutic usefulness of nitric oxide donors in SSc. In both groups, ccIMT correlated with age, in addition, in SSc we demonstrated a correlation between ccIMT and disease duration, although ccIMT of SSc patients was not different from IMT of controls. These findings indicate that accelerated atherosclerosis is not a hallmark of systemic sclerosis.

Screening AIx and PWV over the brachial artery by an automated oscillometric method, we found both parameters elevated in SSc compared to controls. Increased PWV also correlates with age, disease duration, and AIx in patients with SSc. We found PWV of the limited SSc group to be greater than PWV of patients with diffuse SSc. Age-dependency of PWV has previously been reported by numerous studies in the general population, and elevated PWV has also been linked to increased cardiovascular risk. Thus, patients with increased PWV might be candidates for closer cardiovascular follow-up in the clinical setting.

In our third, longitudinal case-series study we assessed the effects of rosuvastatin (20mg/die, for 6 months) on endothelial dysfunction, arterial stiffness, immuno-inflammatory laboratory markers and cardiac abnormalities in SSc. Rosuvastatin improved FMD, decreased hs-CRP, complement levels and immune complex production in SSc, indicating lower disease activity and perhaps reduced cardiovascular risk. We demonstrated that the improvement in endothelial dysfunction is unrelated to the reduction in atherogenic index, which implies a mechanism of action other than the lipid-lowering effect of rosuvastatin. 6-month rosuvastatin treatment had no effect on the ankle-brachial index, on carotid plaques or ccIMT, on arterial stiffness or on forearm cutaneous microcirculation assessed by laser Doppler in SSc. By resting ECG and 24-hour ECG monitoring we evaluated functional cardiac involvement of patients and found a 23% frequency of conduction disturbances, 42% frequency of clinically significant arrhythmias, and a 23% frequency of abnormal heart rate variability in Hungarian SSc patients. Resting conventional and tissue Doppler echocardiography revealed that left ventricular dysfunction is very common in SSc (79.5%), and, similarly to ECG abnormalities or markers of right ventricular function, is not influenced by 6-month rosuvastatin therapy.

In summary, rosuvastatin had a favourable effect on endothelial function and immuno-inflammatory markers CRP, complement and immune complex levels, yet conveyed no change in cardiac and arterial stiffness parameters or physical fittness in SSc, perhaps due to treatment duration, chemical structural causes (such as low lipophylicity), or statin doses. In order to define the place of rosuvastatin in the therapeutic regimen in SSc, however, additional studies are needed in the near future.

9. ÖSSZEFOGLALÁS

A szisztémás sclerosis (SSc) az egyik legrosszabb prognózisú a szisztémás autoimmun kórképek közül, melynek lefolyását egyrészt a belső szervi manifesztációk, másrészt SSc-től független kórképek határozzák meg. Napjainkban a halálozás mintegy harmadáért kardiovaszkuláris okok felelősek sclerodermában. Ezen eltérések megismerése kiemelkedő jelentőségű az SSc-s betegek gondozása szempontjából.

Munkánk során elsőként két keresztmetszeti, kontrollált vizsgálatban tanulmányoztuk az endotélfunkciót (flow-mediált vazodilatáció, FMD), a korai atherosclerosist jelző carotis intimavastagodás jelenlétét (a. carotis communis intima-media vastagság, ccIMT) illetve az érfali merevség paramétereit (pulzushullám-terjedési sebesség, PWV, augmentációs index, AIx mérés) egy-egy SSc-s betegcsoportban. Az FMD-t szignifikánsan alacsonyabbnak találtuk SSc-ben, míg az NMD nem mutatott eltérést a kontrollcsoporthoz képest. A carotis communis IMT-t vizsgálva nem találtunk szignifikáns különbséget a két csoport között, ez a paraméter mindkét csoportban a korral, ezen kívül SSc-ben a betegségtartammal mutatott lineáris összefüggést. Az életkornak megfelelő ccIMT SSc-ben arra utal, hogy szemben más szisztémás autoimmun betegségekkel, ennek a kórképnek nem meghatározó vonása az akcelerált atheroscerosis. A betegségtartammal való összefüggés alapján az SSc-nek is lehetnek azonban olyan aspektusai, melyek, legalábbis a betegek egy részében az atherosclerosis progressziója irányában hatnak. Eredményeink alapján az endotélfunkció romlása korai, funkcionális eltérésként jelentkezik SSc-ben, míg a nitrát-mediált vazodilatáció megtartott, aminek, különösen fiatalabb életkorban, terápiás jelentősége lehet.

Az artériás stiffness indikátorai közül mindkét paramétert kórosan emelkedettnek találtuk SSc-ben. Az életkor és PWV közt fennálló korreláció az átlagpopulációban már ismert volt, azonban vizsgálatunkkal SSc-s betegcsoportban is kimutattuk ezt az összefüggést, valamint a PWV a betegségtartammal is korrelált SSc-ben. Ez alapján célszerűnek tűnik az emelkedett PWV-jű betegek szorosabb követése a sclerodermás betegek vaszkuláris gondozási tervének felállítása során.

Harmadik, longitudinális vizsgálatunkban rosuvastatin hatását vizsgáltuk SSc-ben az immuno-inflammatorikus laboreltérésekre, valamint a kardiovaszkuláris paraméterekre. Azt találtuk, hogy a rosuvastatin javítja az FMD, csökkenti a hs-CRP valamint a komplement szinteket, és csökkenti az immunkomplex termelődést SSc-ben, melyek a betegség aktivitásának, s egyben valószínűleg a betegek kardiovaszkuláris rizikójának csökkenésére utalnak. Kimutattuk, hogy az endotélfunkció romlása nem korrelál az atherogén indexben észlelt változással, azaz a hatásmechanizmus a rosuvastatin lipidcsökkentő hatásától független. Eredményeink alapján 6 hónapos rosuvastatin kezelésnek nem volt hatása a perifériás érbetegség vagy az a. carotis plakkok előfordulási gyakoriságára, a ccIMT-re, az artériás stiffness paraméterekre vagy az alkar bőrének laser Dopplerrel mért microcirculatiójára. Nyugalmi és 24 órás Holter EKG vizsgálat alapján a vezetési zavarok (23%), klinikailag jelentős arrhythmiák (42%), illetve a kóros szívfrekvencia-variabilitás (23%) gyakoriságát mértük fel betegeinknél, s azt a nemzetközi adatokkal nagyjából egyezőnek találtuk. Echocardiographiás vizsgálataink során igen magas, 79.5%-os arányban észleltünk bal kamrai diastolés diszfunkciót SSc-ben, és kimutattuk, hogy 6 hónapos rosuvastatin kezelés sem az EKG eltérésekre, sem a bal kamra diastolés funkcióra, sem a jobb kamra funkcióra nem volt hatással az általunk vizsgált betegcsoportban. Összefoglalva, a rosuvastatin kedvező hatással volt az endotélfunkcióra és immuno-inflammatorikus markerekre, azonban nem volt hatással a cardialis és artériás stiffness paraméterekre SSc-ben. Ahhoz azonban, hogy a rosuvastatin helyét megállapítsuk SSc-ben a terápiás palettán, még további vizsgálatok szükségesek.

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11. LIST OF PUBLICATIONS

Candidate: Orsolya Tímár
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Doctoral School: Doctoral School of Clinical Medicine

List of publications related to the dissertation

1. **Tímár, O.**, Szekanecz, Z., Kerekes, G., Végh, J., Oláh, A.V., Nagy, G., Csiki, Z., Dankó, K., Szamosi, S., Németh, Á., Soltész, P., Szűcs, G.: Rosuvastatin improves impaired endothelial function, lowers high sensitivity C-reactive protein, complement and immuncomplex production in patients with systemic sclerosis: A prospective case-series study.
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4. Soltész, P., Kerekes, G., Dér, H., Szűcs, G., Szántó, S., Kiss, E., Bodolay, E., Zeher, M., **Tímár, O.**, Szodoray, P., Szegedi, G., Szekanecz, Z.: Comparative assessment of vascular function in autoimmune rheumatic diseases: Consideration of prevention and treatment.
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12. KEYWORDS

Keywords: systemic sclerosis, cardiovascular, flow-mediated dilation, arterial stiffness, pulse-wave velocity, rosuvastatin, CRP, ECG, echocardiography

Tárgyszavak: szisztémás sclerosis, kardiovaszkuláris, artériás stiffness, flow-mediált vazodilatáció, pulzushullám-terjedési sebesség, rosuvastatin, CRP, ECG, echocardiographia

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