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PAPERS I-V
Abstract
In systemic sclerosis (SSc), interstitial lung disease (ILD) and engagement of the vascular system lead to increased morbidity and mortality.

The aim of this thesis was to elucidate, in a consecutively included cohort of SSc (limited and diffuse) patients (n = 33), the T cell cytokine profile driving the disease in ILD and to explore the role of matrix metalloproteinase 9 (MMP-9) and its inhibitor: tissue inhibitor of metalloproteinase 1 (TIMP-1) in the extracellular matrix (ECM) degrading process leading to fibrous scarring and honey combing. Moreover, to characterize the role of nitric oxide (NO) in vascular engagement.

Peripheral arterial changes cause Raynaud’s phenomenon and digital ulcers. Nitric oxide (NO) a main inducer of vasodilation is produced by endothelial nitric oxide synthase (eNOS) in response to changes in blood flow or by inflammatory cytokine inducible (i) NOS. In the vascular smooth muscle cell (VSMC) NO activates guanylate cyclase to produce cGMP, causing relaxation. We showed elevated plasma nitrate, a degradation product of NO, and increased urinary excretion of nitrate and cGMP. Plasma nitrate correlated with elevated levels of endothelial adhesion molecules: endothelial (E) selectin and vascular adhesion molecule 1, indicating that the activated endothelium is the site of NO synthesis by iNOS. Endothelial staining for E-selectin and the finding of iNOS and eNOS in SSc skin biopsies supported this notion.

In SSc increased vascular stiffness may limit the NO vasodilatory effects. We found normal endothelium-dependent (i.e. flow mediated (FMD%)) and endothelium-independent (i.e. nitroglycerin-induced (NTG%)) vasodilation in the brachial artery. Radial arterial wall stiffness measured as maximum increase in pulse pressure (dP/dt\textsubscript{max}) was increased. FMD% and especially NTG% correlated negatively and dP/dt\textsubscript{max} positively to measures of endothelial inflammation: plasma- nitrate and adhesion molecule levels. Thus inflammatory vascular wall changes may interfere with dilation as may the presence of nitrate tolerance.

We found elevated alveolar MMP-9 in both its pro- and active form in ILD. The levels correlated to decline in lung capacity, pointing at a causal relation. We suggest that neutrophils secrete MMP-9, which may degrade collagen IV, (the main constituent of basal membranes), collagen V, gelatins, proteoglycans and elastin. MMP-9 activity is partly regulated by the binding of pro- and active form to TIMP-1. Alveolar TIMP-1, which even stimulates fibroblast ECM synthesis, was increased independent of ILD.

The inflammatory process in ILD is orchestrated by activated T helper (h) lymphocytes. We found a mixed Th1/Th2 reaction in SSc alveolar T cells expressing messenger for interferon gamma (Th1), IL-6 and IL-10 (both Th2). No particular cytokine mRNA profile distinguished alveolar T cells in ILD. Neutrophils invaded the bronchial epithelium, which seemed otherwise inert as levels of inflammatory cytokine sensitive transcription factors and their nuclear translocation tended to be low. The neutrophil recruitment pathway is uncertain as chemoattractants and endothelial adhesion molecules were normally expressed.

In conclusion, MMP-9 probably causes degradation of lung tissue in ILD and may represent a future therapeutic target. Alveolar T cells show a mixed Th1/Th2 cytokine profile independent of ILD. Neutrophils invade the bronchial epithelium. Activated endothelium produces increased amounts of NO and adhesion molecules and the level of activation influences brachial arterial FMD% and NTG% and radial arterial compliance. Nitrate tolerance may be present.

**Key words:** Systemic sclerosis, interstitial lung disease, MMP-9, Th1/Th2, transcription factors, NO, iNOS, E-selectin, endothelium-dependent dilation, endothelium-independent dilation.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Definition</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACA</td>
<td>anti-centromere antibodies</td>
</tr>
<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
</tr>
<tr>
<td>Anti-RNP</td>
<td>anti ribonucleoprotein antibodies</td>
</tr>
<tr>
<td>BALF</td>
<td>broncho-alveolar lavage fluid</td>
</tr>
<tr>
<td>BNP</td>
<td>brain natriuretic protein</td>
</tr>
<tr>
<td>CD</td>
<td>cluster of differentiation</td>
</tr>
<tr>
<td>CTGF</td>
<td>connective tissue growth factor</td>
</tr>
<tr>
<td>DL&lt;sub&gt;CO&lt;/sub&gt;</td>
<td>diffusion capacity for carbon monoxid</td>
</tr>
<tr>
<td>dSSc</td>
<td>diffuse systemic sclerosis</td>
</tr>
<tr>
<td>ECM</td>
<td>extracellular matrix</td>
</tr>
<tr>
<td>ECP</td>
<td>eosinophilic cationic protein</td>
</tr>
<tr>
<td>ENA-78</td>
<td>epithelial neutrophil-activating protein 78</td>
</tr>
<tr>
<td>eNOS</td>
<td>endothelial nitric oxide synthase</td>
</tr>
<tr>
<td>E-selectin</td>
<td>endothelial selectin</td>
</tr>
<tr>
<td>F:M</td>
<td>female to male ratio</td>
</tr>
<tr>
<td>FMD</td>
<td>flow mediated dilation</td>
</tr>
<tr>
<td>FVC</td>
<td>forced vital capacity</td>
</tr>
<tr>
<td>GRO&lt;sub&gt;α&lt;/sub&gt;</td>
<td>growth related oncogene alpha</td>
</tr>
<tr>
<td>HRCT</td>
<td>high resolution computed tomography</td>
</tr>
<tr>
<td>ICAM-1</td>
<td>intercellular adhesion molecule 1</td>
</tr>
<tr>
<td>IFN</td>
<td>interferon</td>
</tr>
<tr>
<td>IL</td>
<td>interleukin</td>
</tr>
<tr>
<td>ILD</td>
<td>interstitial lung disease</td>
</tr>
<tr>
<td>iNOS</td>
<td>inducible nitric oxide synthase</td>
</tr>
<tr>
<td>IPHT</td>
<td>isolated pulmonary hypertension</td>
</tr>
<tr>
<td>ISSc</td>
<td>limited systemic sclerosis</td>
</tr>
<tr>
<td>MBP</td>
<td>major basic protein</td>
</tr>
<tr>
<td>MCTD</td>
<td>mixed connective tissue disease</td>
</tr>
<tr>
<td>MMP</td>
<td>metalloproteinase</td>
</tr>
<tr>
<td>MPO</td>
<td>myeloperoxidase</td>
</tr>
<tr>
<td>NFKB</td>
<td>nuclear factor kappa B</td>
</tr>
<tr>
<td>NE</td>
<td>neutrophil elastase</td>
</tr>
<tr>
<td>NO</td>
<td>nitric oxide</td>
</tr>
<tr>
<td>NO&lt;sub&gt;2&lt;/sub&gt;</td>
<td>nitrite</td>
</tr>
<tr>
<td>NO&lt;sub&gt;3&lt;/sub&gt;</td>
<td>nitrate</td>
</tr>
<tr>
<td>ONOO-</td>
<td>peroxynitrite</td>
</tr>
<tr>
<td>PAP</td>
<td>pulmonary arterial pressure</td>
</tr>
<tr>
<td>PBMC</td>
<td>peripheral blood mononuclear cells</td>
</tr>
<tr>
<td>PDE</td>
<td>phosphodiesterase</td>
</tr>
<tr>
<td>PDGF</td>
<td>platelet derived growth factor</td>
</tr>
<tr>
<td>PTK</td>
<td>protein tyrosine kinase</td>
</tr>
<tr>
<td>P-selectin</td>
<td>platelet selectin</td>
</tr>
<tr>
<td>Scl-70 ab</td>
<td>scleroderma 70 antibodies</td>
</tr>
<tr>
<td>SCF</td>
<td>stem cell factor</td>
</tr>
<tr>
<td>SD</td>
<td>standard deviation</td>
</tr>
<tr>
<td>SRC</td>
<td>scleroderma renal crisis</td>
</tr>
<tr>
<td>SSc</td>
<td>systemic sclerosis</td>
</tr>
<tr>
<td>Tc</td>
<td>T cytotoxic cell</td>
</tr>
<tr>
<td>TGF&lt;sub&gt;β&lt;/sub&gt;</td>
<td>transforming growth factor beta</td>
</tr>
<tr>
<td>Th</td>
<td>T helper cell</td>
</tr>
<tr>
<td>TIMP</td>
<td>tissue inhibitor of metalloproteinase</td>
</tr>
<tr>
<td>TLC</td>
<td>total lung capacity</td>
</tr>
<tr>
<td>TNF</td>
<td>tumor necrosis factor</td>
</tr>
<tr>
<td>Treg</td>
<td>T regulatory cell</td>
</tr>
<tr>
<td>VC</td>
<td>vital capacity</td>
</tr>
<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
</tr>
<tr>
<td>VCAM-1</td>
<td>vascular adhesion molecule 1</td>
</tr>
<tr>
<td>VSMC</td>
<td>vascular smooth muscle cell</td>
</tr>
</tbody>
</table>
ORIGINAL PAPERS


INTRODUCTION

SYSTEMIC SCLEROSIS

Definition Systemic sclerosis (SSc) is a generalised disorder, characterised by scarring (fibrosis) and vascular obliteration in the skin, gastrointestinal tract, lungs, heart and kidneys, in which hidebound skin is the clinical hallmark and the degree of organ involvement predicts prognosis. The disease spectrum ranges from diffuse SSc with widespread thickening of the skin with rapidly progressive, severe visceral involvement, to a form with limited skin changes, in which several decades may elapse before expression of internal manifestations (LeRoy 1988). Also intermediate forms exist, including patients with “overlap” disorders. The CREST syndrome is characterized by calcinosis, Raynaud’s phenomenon, oesophageal dysfunction, sclerodactyly and telangiectasia.

Disease criteria and classification of subsets In an intent to establish classification criteria a multicenter study of early-diagnosed cases of SSc was carried out 1980 and preliminary criteria proposed by a subcommittee of the American Rheumatism Association (Subcommittee of the American Rheumatism Association 1980). Criteria are shown in table 1. The finding of the sole major criterion has a sensitivity of 91% and a specificity of 99.8% and the finding of two or more of the minor criteria carries a sensitivity of 97% and a specificity of 98%, when compared to patients with other collagen disorders.

Table 1. Preliminary criteria for the classification of SSc according to the American Rheumatism Association.

<table>
<thead>
<tr>
<th>Major criteria:</th>
<th>Proximal scleroderma: scleroderma involvement proximal to the digits.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Minor criteria:</td>
<td>1. Sclerodactyly: scleroderma involvement in the fingers and toes.</td>
</tr>
<tr>
<td></td>
<td>2. Digital pitting scars</td>
</tr>
<tr>
<td></td>
<td>3. Bilateral basilar pulmonary fibrosis on chest roentgenogram</td>
</tr>
</tbody>
</table>

Since SSc is a heterogenous disease, many have attempted to establish classification of subsets (Winkelmann 1976, Giordano 1986, Rodnan 1976, Barnett 1988). The classification by LeRoy has proven especially useful in the prediction of disease course and outcome in individual patients. The classification outlines the characteristics and distinctions of two disease subsets in SSc, a larger group with limited (l) SSc and a smaller with diffuse (d) skin involvement (dSSc). The grouping hinges on cutaneous involvement, but is valuable because the two outlined subsets differ as to type and time for visceral involvement, antibodies produced, disease progression and outcome. In this thesis, the classification by LeRoy et al is used (LeRoy 1988).

Table 2. Subsets of systemic sclerosis

<table>
<thead>
<tr>
<th>Diffuse cutaneous SSc (dSSc)</th>
<th>Limited cutaneous SSc (ISSc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Onset of Raynaud’s phenomenon within 1 year of onset of skin changes (puffy or hidebound)</td>
<td>Raynaud’s phenomenon for years (occasionally decades)</td>
</tr>
<tr>
<td>Truncal and acral skin involvement</td>
<td>Skin involvement limited to hands, face, feet, and forearms (acral) or absent</td>
</tr>
<tr>
<td>Presence of tendon friction rubs</td>
<td></td>
</tr>
<tr>
<td>Early and significant incidence of interstitial lung disease, oliguric renal failure, diffuse gastrointestinal disease, myocardial involvement</td>
<td>A significant late incidence of pulmonary hypertension, with or without interstitial lung disease, trigeminal neuralgia, skin calcifications, telangiectasia</td>
</tr>
<tr>
<td>Absence of anticientromere antibodies (ACA)</td>
<td>A high incidence of ACA, (70-80%)</td>
</tr>
<tr>
<td>Nail fold capillary dilation and capillary destruction</td>
<td>Dilated nail fold capillary loops, usually without capillary dropout</td>
</tr>
<tr>
<td>Antitopoisomerase antibodies (anti-Scl-70 antibodies) (30% of patients)</td>
<td></td>
</tr>
</tbody>
</table>

Overlap disorders.
The overlap patients have features of both SSc and other collagen disorders and may represent transitions in the spectrum of SSc and these diseases. One group within the overlap patients have high titers of anti-ribonucleoprotein antibodies (anti-RNP ab) and may evolve the mixed connective tissue disease (MCTD) syndrome (Sharp 1972).

EPIDEMIOLOGY OF SYSTEMIC SCLEROSIS

Incidence and prevalence There are only a few prospective studies on the incidence and prevalence of SSc (Geirsson 1994, Roberts-Thomson 2001). In retrospective studies data may be biased, as severely ill patients may die before referral and less severe not be included. Thus dSSc:ISSc ratio may be inadvertently high as may the male:female ratio as dSSc is more common in men (Hesselstrand 1998).
In two retrospective studies in the USA, the incidences reported were about 18 new cases per million (table 3) (Steen 1997, Mayes 2003), which was in agreement with the incidence found prospectively in southern Australia. The prevalence was estimated to be at least 225 cases per million in white Americans (Mayes 2003) and 233 in South Australians (Roberts-Thomson 2001). In Iceland a low incidence of 3.8 per million may be due to a unique gene composition in the population (Geirsson 1994).

**Female to male ratio** The incidence and prevalence in females is significantly greater than in men especially in ISSc (table 3). The greater prevalence than incidence female to male (F:M) ratio reflects an increased mortality in male SSc patients and in men in general (Mayes 2003). The peak F:M incidence ratio in white patients (4:1) is in the age group 35-44 years (Steen 1997).

**Prevalence according to disease subsets** From the numbers given by Mayes et al 2003, a prevalence for ISSc of 165 and for dSSc of 60 per million whites in the USA may be deduced.

**Age at peak incidence** Reported age at the incidence peak may vary partly due to differences in the definition of disease onset, whether it is defined as the appearance of the first symptom including Raynaud’s phenomenon (Geirsson 1994, Steen 1997) or first non-Raynaud’s symptom (Mayes 2003) (table 3).

### Table 3. Epidemiology in systemic sclerosis

<table>
<thead>
<tr>
<th>First author-year</th>
<th>Geographical area</th>
<th>Time period</th>
<th>Type of investigation</th>
<th>Patient number</th>
<th>Female : Male incidence ratio</th>
<th>Incidence: new cases/million/year</th>
<th>Prevalence: cases/million</th>
<th>Age at peak incidence (yrs)</th>
<th>Mean age at onset (yrs)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Geirsson 1994, Iceland</td>
<td></td>
<td>1975-90</td>
<td>Prospective from 1980</td>
<td>18 all 13 ISSc 5 dSSc</td>
<td>14</td>
<td>3.8 in all 7 in females 0.5 in males</td>
<td>71 in all 119 in females 15 in males</td>
<td>40-49 in all</td>
<td>43.8</td>
</tr>
<tr>
<td>Steen 1997, Pennsylvania, USA</td>
<td></td>
<td>1963-82</td>
<td>Retrospective</td>
<td>444 all 405 definite 39 probable</td>
<td>3.1 in all 3.2 in whites 2.7 in blacks</td>
<td>13.9 (1963-82) 9.6 (1963-72) 18.7 (1973-82) 13.3 (1963-72 F) 27.6 (1973-82 F)</td>
<td>45-55 B F 55-64 W F</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mayes 2003, Detroit</td>
<td></td>
<td>1989-92</td>
<td>Retrospective (22% blacks)</td>
<td>706 all 595 definite 111 probable 308 ISSc (46 blacks) 165 dSSc (70 blacks)</td>
<td>3.2 (incidence) 4.6 (prevalence)</td>
<td>19.3 in all 23.7 in blacks 18.3 in whites</td>
<td>242 in all 390 in females 84 in males</td>
<td>65-74 W F 45-54 B F 75-84 W M 45-54 B M</td>
<td></td>
</tr>
</tbody>
</table>

F = female, M = male, B = blacks, W = whites

**5- and 10 years survival** Survival may be estimated from the first self-reported symptom or from time of diagnosis. In a retrospective study starting at the point of diagnosis, survival from the first symptom up to this point will be 100% and survival overestimated. Survival may increase due to new treatment options, like the availability of angiotensin converting enzyme (ACE) inhibitors for the treatment of scleroderma renal crisis (SRC) from 1980, and cyclophosphamide (CYC) for the treatment of interstitial lung disease (ILD) from 1990. Survival may seemingly improve because of the selection of still more patients at an earlier disease state and with less severe disease. 5- and 10-year survivals from disease onset in a Swedish (Hesselstrand 1998) and an English study (Bryan 1996), describing the time period 1982-1995 are very similar, that is 5-year survival of 92% and 87%, respectively; 10-year survival of 78% and 75%. In a Danish study on patients diagnosed 1960-96, survival from time of diagnosis, was somewhat lower (Jacobsen 1998), probably due to a later inclusion point and lack of efficient treatment of SRC until 1980.

**Mortality in relation to disease subsets** In almost all studies, mortality measured by the standardised mortality rate (SMR) has been found significantly increased in SSc, with SMRs from 2.3 in ISSc to 6.2 in dSSc. SMR in dSSc is in several studies significantly higher than in ISSc (Abu-Shakra 1995, Jacobsen 1998, Hesselstrand 1998), a finding, however, not confirmed by Mayes et al 2003.
Causes of death In an SSc population diagnosed 1960-96, 41% of deaths were due to renal failure and 31% to lung engagement (Jacobsen 1998). After the advent of ACE inhibitors the dominating cause of death in SSc is consequences of ILD i.e. respiratory failure and bronchopneumonia. Thus 36-44% of SSc related deaths were due to ILD in SSc populations with disease onset 1982-1995 (Bryan 1996, Hesselstrand 1998). In the study by Jacobsen et al 1998, the number of unrelated deaths was about twice the expected, a finding supported by other studies. The findings suggest unrecognized vascular heart disease in early SSc.

DISEASE MANIFESTATIONS IN SSc

Clinical skin changes Skin engagement goes through three stages: a first stage of oedema, then indurated thickening and finally thinning and atrophy. Typically, symmetrical skin changes begin on the fingers and spread centrally, the last area to be involved is the first to show regression. Severity may be monitored by serial Rodnan score estimations (Silman 1995). Small macular punctuate telangiectasias composed of dilated postcapillary venules may appear on the hands and face. In ISSc calcifications may develop in locations subjected to external pressure, like fingertips, knees and elbows.

Histopathology of the skin lesion The dermal lesion is probably the most thoroughly investigated lesion in SSc because of the easy accessibility, however, the pathogenesis of the lesion in other organs is probably very similar. Skin biopsies from SSc patients typically show thickening of the dermis due to the deposition of bundles of collagen fibres. Inflammatory cells infiltrate the perivascular areas, especially on the boundary between dermis and subcutis. The walls of the small arteries may be thickened and calcifications may be encountered.

Endothelial cells The skin lesion in SSc may start with endothelial cell activation, leading to inflammatory cell extravasation and fibroblast activation. Immunohistochemical staining has shown that the dermal endothelial cell is activated and expresses adhesion molecules (Sollberg 1992, class II major histocompatibility complex (MHCII) (Gruschwitz 1997), transforming growth factor (TGF)β (Gabrielli 1993), the vasoconstrictor and fibroblast mitogen: endothelin (Vancheeswaran 1994) as well as inducible nitric oxide synthase (iNOS) (Cotton 1999).

Leukocytes T cells, B-cells, monocytes/macrophages, mast cells and eosinophils infiltrate the skin in SSc. According to one theory, antigen-driven T cell activation and clonal expansion are the primary events and the disease process driven by T cell-derived cytokines. Both T cells bearing the αβ- and the γδ T cell receptor are present and oligoclonally expanded, probably reacting to the presentation of their specific activating (auto?) antigen (Giacomelli 1998, Sakkas 2002). The T helper (Th) 2 reaction may dominate, as the skin seems invaded by Th cells expressing CD30, secreting interleukin (IL) -4 and 5 (Mavilia 1997) and by double CD4/CD8 positive T cells also producing IL-4 (Parel 2007). Interleukin 4 and 5 activate invading B cells to express high levels of CD19 antigen and receptors for the B cell activating factor (BAFF) (Matsushita 2006) as well as to switch to IgE and high-affinity IgG production. IL-5 attracts mast cells and eosinophils, which in turn may augment the activation of fibroblasts, started by IL-4. Interferon (IFN) γ and tumor necrosis factor (TNF) α produced by Th 1 cells (Yamane 2003, Gruschwitz 1997), or membrane-associated TNFα on Th2 cells may act to oppose these effects and inhibit collagen production (Chizzolini 2003). Invading Th17 cell may induce endothelial cell adhesion molecule expression, important to the recruitment of lymphocytes and stimulate fibroblast proliferation (Kurasawa 2000).

Mast cells invading the SSc skin lesion (Seibold 1990), are activated by binding of IgE (Ohtsuka 2005) and degranulate, releasing histamine and chymase in affected as well as in areas with involvement to come (Irani 1992), which may promote the edematous stage. Experimentally, mast cell histamine and tryptase stimulate fibroblast production of collagen and tissue inhibitor of metalloproteinase (TIMP) furthering fibrosis (Garbuzenko 2001).

Fibroblasts IL-4 and TGFβ transform the SSc skin fibroblast into an activated alpha-actin harbouring myofibroblast, which produces excessive amounts of extracellular matrix (ECM) constituents: collagen I, III, IV, V, VI and VII, fibronectin, glycosaminoglycans: dermatan- and chondroitin sulphates and decorin in SSc (Kuroda 1997). Moreover, SSc skin fibroblasts produce increased amounts of TIMP-1, and decreased amounts of metalloproteinases (MMPs), leading to the attenuation of ECM remodelling (Kirk 1995). TGFβ binds to a complex of TGFβ receptor I and II, which signal through the smad pathway. Abnormalities of the smad system may be responsible for the increased transcription of ECM genes in SSc skin (Varga 2002, review) as may an increased expression of the TGFβ RI and II (Yamane 2002). TGFβ even stimulates fibroblasts to secrete connective tissue growth factor (CTGF), which further stimulates fibroblast collagen production (Shi-Wen 2000). Several autocrine loops exist, thus the activated fibroblasts secrete IL-4, TGFβ and TIMP-1 (Kikuchi 1997, Kawakami 1998).
THE INVOLVEMENT OF DIFFERENT CELL TYPES IN SSC PATHOGENESIS.

SSc skin is invaded by activated T helper cells producing IL-4, IL-5, IL-13, IL-17 and TGFβ. The invasion of T cells is promoted by the adhesion to activated endothelial cells in the blood stream. IL-4 and 5 stimulate B-cell maturation into (auto) antibody producing plasma cells. IL-4 and TGFβ stimulate fibroblasts to extracellular matrix formation, TIMP-1 and CTGF synthesis. CTGF = connective tissue growth factor; ICAM-1= intercellular adhesion molecule 1; IL = interleukin; PDGF = platelet derived growth factor; TGF = transforming growth factor beta. VCAM-1 = vascular adhesion molecule 1.

Modified from Zuber JP. Rheumatology 2006;suppl3:iii23-5

INVOLVEMENT OF THE GASTROINTESTINAL TRACT

In both subtypes of SSc, the gastrointestinal tract is extensively involved. The histopathology is characterized by fibrosis of the muscular stratum with deposition of collagen and elastic fibres around smooth muscle cells and invasion of lymphocytes, neutrophils, eosinophils and mast cells, while vascular walls may be thickened and vessels dilated or occluded. In the stomach and intestines “water melon” changes, with linear watermelon-like ectatic vascular stripes, may be found.

Oesophagus and stomach The smooth muscular distal 2/3rd of the oesophagus is involved in 80% of SSc patients, leading to dysmotility. The lower oesophageal sphincter is often patent and gastric wall compliance reduced due to fibrosis, resulting in gastro - oesophageal reflux and impaired migrating motor complex. Myofibroblasts reside in the gastric wall lamina propria, where increased amounts of collagens I, III and IV and fibrogenic cytokines: TGFβ, CTGF and endothelin have been shown (Manetti 2007).

Smaller and greater intestine Reduced small bowel motility is common and favours colonic bacterial overgrowth, malabsorption and diarrhoea. Pneumatosis cystoides intestinalis is a rare complication with multiple air-filled cysts in the submucosa and subserosa, which ruptured cause pneumoperitoneum. Dilatation of the colonic wall, loss of haustration and presence of wide-mouthed diverticula result in prolonged transit time. Anorectal dysfunction due to fibrosis of the internal smooth muscle sphincter is common (Ebert 2008, review).

CARDIAC INVOLVEMENT

Cardiac involvement in SSc includes myocardial, pericardial and conduction system disease. Myocardial engagement with patchy fibrosis, caused by concentric intimal hypertrophy of the intramyocardial small arteries, seems to be a distinct pathologic entity in SSc. Thus at autopsy, myocardial fibrosis and lesions of the small coronary arteries and arterioles are significantly more common in young SSc patients (D’Angelo 1969). A high prevalence of myocardial contraction band fibrosis, which may be due to reperfusion injury has been described (Bulkley 1976). The fibrosis of the
myocardium may lead to hypertrophy of the septum and left ventricular, posterior wall, resulting in decreased compliance with impaired left ventricular diastolic filling, increased systolic emptying time and secondary atrial hypertrophy in 60-80% of dSSc patients (Kazzam 1990, 1991) and is related to disease severity and skin score (Follansbee 1984, Nakajima 2006). It seems that the fibrogenic endothelin may be engaged in the pathogenesis of myocardial fibrosis (Kazzam 1997). A high incidence of cold- and stress induced myocardial reversible ischemia has been demonstrated, especially in dSSc (Gustafsson 1989, Vacca 2006). Signs of pericarditis are found in more than 50% at autopsy (D’Angelo 1968). Arrhythmias and electrocardiographic conduction disturbances are frequent, but seldom of clinical significance (Ridolfi 1976).

**RENAL ENGAGEMENT**

Vascular changes are almost universal in SSc and the histopathology very similar in different vascular beds, characterized by intimal thickening, lumen reduction, endothelial cell proliferation and crescent formation, folding and rupture of the elastica interna, medial thickening with fibrinoid deposition and adventitial scarring (figure 2).

Typical histopathologic changes are common in small, medium sized and large renal arteries in dSSc and also occur in small renal arteries (arterioles) in ISSc, but are most pronounced in dSSc patients with SRC (Trostle 1988). Renal vascular function is impaired, rendering SSc patients unable to increase glomerular filtration rate and to decrease renal vascular resistance upon challenge (Livi 2006). Doppler-flow studies show increased renal vascular resistance, most pronounced in dSSc patients with Scl-70 antibodies (Nishijima 2001).

SRC is defined as the occurrence of malignant arterial hypertension, (diastolic blood pressure above 120 mm Hg with grade III-IV hypertensive retinal changes) or rapidly progressive oliguric renal failure (Steen 1984). Marked hyperreninemia and hyperaldosteronism are almost universal findings, but SRC is not heralded by sudden rise in renin and aldosterone levels (Steen 1984). SRC develops in about 15-20% of dSSc patients, rarely in ISSc (about 1%), a mean of 1.7 years after disease onset (DeMarco 2002). Rapid spread of skin thickening precedes SRC. Congestive heart failure and the initiation of moderate-dose prednisolone medication may provoke SRC onset (Steen 1998). Before ACE inhibitor therapy became available in 1980, SRC had a 1-year survival of 15%, of whom 2/3 needed permanent dialysis. Captopril therapy resulted in a 1-year survival of 75% and a 5-year survival similar to that found in dSSc patients without SRC (Steen 1990).

**PULMONARY HYPERTENSION**

Histopathologic changes of the arteriolar intima and media are found in 29% of autopsy cases (D’Angelo 1969). Morphometric studies on lung sections show the presence of changes in both disease subsets, except in dSSc succumbed to SRC, and most pronounced in ISSc with isolated pulmonary hypertension (IPHT). Thus intimal area and luminal occlusion is increased in small- and especially in medium sized and large arteries, in which also medial thickness is increased (Al-Sabbagh 1969). Also pulmonary vascular function is abnormal as endothelium dependent dilation in ISSc, measured as increase in cardiac output after stimulation with substance P is impeded (Cailles 1998).

IPHT, that is mean pulmonary arterial pressure (PAP) > 20 mm Hg and systolic/diastolic pressures > 30/15 mm Hg in patients without ILD, occurs in about 5-10% of ISSc patients with > 14 years disease duration (Stupi 1986, Steen 2003); However, pulmonary hypertension due to similar vascular changes as in IPHT appear in patients with ILD (Chang 2003), yet, the interstitial lung changes may contribute to the raised PAP (Hesselstrand 2005) as pressure increases with restrictivity. An initially elevated PAP (Chang 2006, Hesselstrand 2005) implies a greater risk for developing PHT. While diffusion capacity for carbon monoxide (DLCO) < 50% of predicted, higher age at disease debut, Raynaud’s phenomenon as debutting symptom (Stupi 1986), and the absence of Scl-70 antibodies (Steen 2003) imply a greater risk of developing IPHT, male gender, full-blown disease at first examination and the presence of anti-U3RNP antibodies imply a greater risk of developing PHT in the setting of ILD (Morelli 1997). To screen for early IPHT in SSc patients with exertional dyspnoea, echocardiography with or without stress test is preferable (Mukerjee 2004). However, to diagnose treatment requiring PHT, right-sided hearth catheterization is the golden standard. Pulmonary hypertension isolated or in combination with ILD has equally poor prognosis (Chang 2003) with a median survival of 12 months and a 2 year mortality of 50-60% (Stupi 1986, Kawut 2003). However, new treatment options like prostacyclin analogues, the endothelin receptor blocker bosentan and phosphodiesterase (PDE) 5 inhibitors have improved survival.
VASCULAR HISTOPATHOLOGY IN SYSTEMIC SCLEROSIS

Lung section stained with van Gieson elastin stain, which colours elastic tissue black and blood yellow. In these two pulmonary arterioles muscle fibers of the hypertrophied media have been replaced by fibrous tissue. The internal elastic lamina is intact and appears to have been augmented by wavy black staining fibrils. The intima (left arteriole) is very thick, consisting of cellular connective tissue, so that the lumen is greatly reduced. The other artery (right) shows mild intimal proliferation. There is mild capillary congestion. The alveoli and interstitial tissue show no signs of fibrosis.

Kidney section stained with haematoxylin -eosin. The media of the small interlobular artery (centre) is fibrous, the intima is thickened with edematous, myxoid concentric proliferation, which almost totally obliterates the lumen. The glomerulus (top left) is intact and surrounded by normally appearing convoluted tubuli. There is slight interstitial fibrosis.


ENDOTHELIAL FUNCTION IN SYSTEMIC SCLEROSIS

Introduction The peripheral vasculature in SSc is characterized by both structural vascular wall changes and functional changes in the response to shear stress, temperature and vasodilator/constrictor stimulation. Ischemia reperfusion injuries caused by cold- or sympathetic activity provoked vasospasm may lead to vascular lesions. Thus Raynaud’s phenomenon may result in increased oxidant stress, which in turn may lead to endothelial injury and subsequent vascular and extra-vascular damage. Furthermore, there is experimental evidence that steady laminar flow – as opposed to ischemia-reperfusion – has beneficial effects on endothelial cell function, inhibiting adhesion molecule expression and vascular smooth muscle cell (VSMC) proliferation and increasing endothelial cell survival (Skilbeck 2004, Tsai 2007).

Raynaud’s phenomenon Raynaud’s phenomenon, caused by spasm in the small digital arteries, is characterized by a triphasic colour change: the digits become white, then blue and finally turn into a post-occlusion hyperaemic red stage. Raynaud’s phenomenon, triggered by cold, emotional and other stimuli is present in 1-20% of the general population (Maricq 1993), but only 3-20% of these persons have SSc spectrum symptoms (Gerbracht 1985). Raynaud’s phenomenon is equally frequent in ISSc and dSSc and may lead to digital ulceration and gangrene. While in dSSc, Raynaud’s phenomenon is closely related to other debuting symptoms, in ISSc, Raynaud’s phenomenon precedes other disease symptoms including skin changes by more than a decade. Frequency, duration and severity of Raynaud’s attacks and the presence of digital ulcers in SSc have great impact on the patient’s experience of pain and disability. Severity may be estimated by the use of several instruments including the Raynaud’s Condition Score (Merkel 2002).

Endothelium-dependent dilation (table 4) Vascular engagement and function has been investigated in all regions of the upper extremity with varying techniques in SSc.

Endothelium nitric oxide (NO) release by endothelial nitric oxide synthase (eNOS), in response to different stimuli, results in endothelium-dependent dilation. In order to become an electron-transferring enzyme to produce the radical gas: NO, eNOS must be phosphorylated at serine 1177. Phosphorylation begins with the activation of various endothelial cell surface receptors followed by signal transduction. Thus stimulation of the muscarinic receptor by acetylcholine, the bradykinin receptor or the neurokinin receptor by bradykinin or substance P will lead to phosphorylation as will mechanotransduction via fluid shear stress (Jin 2003). In capillaries, however, the acetylcholine induced dilation partly depends on the release of stored prostacyclin (Warren 1994). Moreover, estrogen may mobilize inactive eNOS harboured in cell membrane pouches, (caveolae) and thus increase NO formation. NO in turn activates VSMC soluble guanylate cyclase to produce cGMP, which causes relaxation through Ca^2+ channel activation.
To examine endothelium-dependent dilation, vasodilatory substances may be given orally, direct intraarterially or into the dermal circulation by iontophoresis, a non-invasive method to introduce ionised substances by means of a minor electric current. NO release by eNOS may also be stimulated by increasing the blood flow (flow mediated dilation, FMD) as can be achieved by temporary occlusion of the circulation distal to the artery examined (reactive hyperemia). Increase in vascular diameter may be measured by high-resolution ultrasound, changes in blood flow and velocity by Doppler flowmetry and imaging and venous occlusion plethysmography.

Table 4. Endothelium-dependent dilation in SSc

<table>
<thead>
<tr>
<th>First author year</th>
<th>Patients controls</th>
<th>Mediator</th>
<th>Region examined</th>
<th>Measuring method</th>
<th>Vasodilation</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wigley 1990</td>
<td>21 SSc 29 ctrls</td>
<td>Ischaemia Carba-prostacyclin</td>
<td>Finger skin</td>
<td>Laser Doppler flowmetry</td>
<td>↓Ischaemia</td>
<td>Defect endothelium</td>
</tr>
<tr>
<td>Anderson 1996</td>
<td>10 SSc 10 ctrls</td>
<td>Ach Ischaemia Forearm skin</td>
<td>Iontophoresis Dual channel laser Doppler</td>
<td>ND Ach</td>
<td>Too few patients? Dysfunctional endothelium?</td>
<td></td>
</tr>
<tr>
<td>Lekakis 1998</td>
<td>12 SSc 12 ctrls</td>
<td>Ischaemia Estrogen Brachial artery</td>
<td>Echo-Doppler ultrasound</td>
<td>↓Ischaemia</td>
<td>Defect endothelium</td>
<td></td>
</tr>
<tr>
<td>La Civita 1998</td>
<td>11SSc 16 ctrls</td>
<td>Ach Ischaemia Finger skin</td>
<td>Iontophoresis Laser Doppler flowmetry</td>
<td>↓↓Ach Ischaemia</td>
<td>Defect endothelium</td>
<td></td>
</tr>
<tr>
<td>Anderson 1999</td>
<td>8 SSc 8 ctrls</td>
<td>Ach Bradykinin Finger skin</td>
<td>Iontophoresis Dual channel laser Doppler</td>
<td>ND Ach</td>
<td>Too few patients? Insensitive method?</td>
<td></td>
</tr>
<tr>
<td>Flavahan 2000</td>
<td>11 dSSc 8 ctrls</td>
<td>Ach Bradykinin Upper arm skin</td>
<td>Perfusion of dissected arterioles</td>
<td>ND Ach</td>
<td>Normal endothelium</td>
<td></td>
</tr>
<tr>
<td>Freedman 2001</td>
<td>10 SSc 11 ctrls</td>
<td>Substance P Bradykinin Finger blood flow</td>
<td>Infusion in brachial artery Venous occlusion plethysmography</td>
<td>Vasocpromotion by both substances</td>
<td>Defect PTK pathway?</td>
<td></td>
</tr>
<tr>
<td>Anderson 2004</td>
<td>10 ISSc 11 ctrls</td>
<td>Ach Finger skin</td>
<td>Iontophoresis Laser Doppler imaging</td>
<td>↓Ach</td>
<td>Defect endothelium Sensitive technique</td>
<td></td>
</tr>
<tr>
<td>Shlez 2003</td>
<td>10 SSc 10 ctrls</td>
<td>Ach Nailfold capillaries</td>
<td>Micropipette technique Video imaging</td>
<td>↓Ach</td>
<td>Defective prostacyclin release</td>
<td></td>
</tr>
<tr>
<td>Furspan 2005</td>
<td>17 SSc 17 ctrls</td>
<td>Ach Forearm skin</td>
<td>Perfusion of dissected forearm arterioles</td>
<td>↓Ach</td>
<td>Defect endothelium</td>
<td></td>
</tr>
</tbody>
</table>

ND= no significant difference; Ach = acetylcholine; PTK=protein tyrosine kinase. △ Compared to controls; * compared to pre-treatment value.

In SSc, endothelial function of the brachial artery seems defect as FMD in response to reactive hyperaemia is decreased, but may be partly restored by estrogen treatment (Lekakis 1998). However, endothelial function in upper arm dermal arterioles is normal in response to acetylcholine and bradykinin (Flavahan 2000). The status of the endothelium in forearm- and digital skin is questioned as experimental results differ, probably due to methodological differences and small numbers of patients examined. Thus, in forearm skin endothelial function in response to acetylcholine seems normal, while FMD is reduced, indicating some degree of endothelial damage (Anderson 1996). In digital skin, increase in blood flow in response to ischemia and acetylcholine stimulation is low, pointing at endothelial dysfunction (Wigley 1990, La Civita 1998, Anderson 2004). A paradox vasocpromotion in digital skin to infused bradykinin and substance P is interpreted as a sign of endothelial protein tyrosine kinase (PTK) pathway disturbance (Freedman 2001). Further experiments on arterioles from forearm skin support the presence of increased PTK activity leading to increased contraction upon cooling (Furspan 2005). Endothelial dependent dilation after acetylcholine injection is impaired even in nailfold capillaries in SSc (Shlez 2003).

In summary, endothelium-dependent dilation, as a measure of endothelial function is decreased in the brachial artery, forearm- and digital skin microcirculation and nailfold capillaries. Endothelium-independent dilation (table 5) Endothelium-independent vasodilation is achieved by stimulation of the VSMC with NO released from nitroprusside or nitroglycerin. The relaxation accomplished is a measure of VSMC function and vascular compliance, i.e. vascular dilatory reserve.
and vascular wall stiffsness, which reflects vascular wall structural changes. By means of this method, the wall of the brachial artery was found structurally altered (Lekakis 1998), while structural changes in the vasculature of forearm skin are minimal in SSc (Anderson 1996), but may be present in digital skin (La Civita 1998). In nailfold capillaries, endothelium-independent dilation seems normal (Schlez 2003).

In summary, these observations point at preserved VSMC- and vascular wall function in SSc forearm skin vessels and nailfold capillaries, and a reduced dilatory reserve in the brachial artery and in digital skin vessels.

**Table 5. Endothelium-independent dilation in SSc**

<table>
<thead>
<tr>
<th>First author year</th>
<th>Patients controls</th>
<th>Mediator</th>
<th>Region examined</th>
<th>Method</th>
<th>Vasodilation*</th>
<th>Interpretation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Andersson 1996</td>
<td>10 SSc 10 ctrls</td>
<td>SNP</td>
<td>Forearm skin</td>
<td>Iontophoresis Dual channel laser Doppler</td>
<td>ND</td>
<td>Too few patients? submaximal dilation?</td>
</tr>
<tr>
<td>Lekakis 1998</td>
<td>12 SSc 12 ctrls</td>
<td>NTG estrogen</td>
<td>Brachial artery</td>
<td>Echo-Doppler ultrasound</td>
<td>↓ ND* Estrogen</td>
<td>Structural wall changes</td>
</tr>
<tr>
<td>La Civita 1998</td>
<td>11 SSc 16 ctrls</td>
<td>SNP</td>
<td>Finger skin</td>
<td>Iontophoresis Laser Doppler flowmetry</td>
<td>↓</td>
<td>Structural wall changes</td>
</tr>
<tr>
<td>Anderson 1999</td>
<td>8 SSc 8 ctrls</td>
<td>SNP</td>
<td>Finger skin</td>
<td>Iontophoresis Dual channel laser Doppler</td>
<td>ND</td>
<td>Too few patients? Insensitive method?</td>
</tr>
<tr>
<td>Freedman 2001</td>
<td>10 SSc 11 ctrls</td>
<td>NSP</td>
<td>Finger blood flow</td>
<td>Infusion in the brachial artery Venous occlusion plethysmography</td>
<td>ND</td>
<td>Unimpaired VSMC responsiveness</td>
</tr>
<tr>
<td>Schlez 2003</td>
<td>10 SSc 10 ctrls</td>
<td>NSP</td>
<td>Nailfold capillaries</td>
<td>Micropipette technique Video imaging</td>
<td>ND</td>
<td>Normal precapillary function</td>
</tr>
<tr>
<td>Anderson 2004</td>
<td>10 ISSc 11 ctrls</td>
<td>SNP</td>
<td>Finger skin</td>
<td>Iontophoresis Laser Doppler imaging</td>
<td>↓</td>
<td>Structural wall changes Sensitive technique</td>
</tr>
</tbody>
</table>

ND= no significant difference; NTG = nitroglycerin; SNP = sodium nitroprusside. * Compared to controls; † compared to pre-treatment value.

**ADHESION MOLECULES IN SYSTEMIC SCLEROSIS**

**Introduction** Adhesion molecules belong to three families: 1) the selectin family comprising endothelial-, leukocyte- and platelet selectin (L- P- and E-selectin), 2) adhesion molecules of the immunoglobulin superfamily to which intercellular adhesion molecule (ICAM) 1 and vascular adhesion molecule (VCAM) 1 belong and 3) the integrin family of which leukocyte function-related antigen (LFA) 1, 2 and very late activation antigen (VLA) 1-5 are members. Adhesion molecules bind to specific ligands: selectins bind to mucin-like glycoproteins, adhesion molecules of the immunoglobulin family to integrins. Endothelial cells and leukocytes express adhesion molecules upon inflammatory cytokine stimulation, primarily via nuclear factor kappa B (NFkB) mediated transcription in order to achieve extravasation (figure 3). All three selectins, VCAM-1, and ICAM-1 exist in soluble form, shed from the activated cell within minutes after stimulation.

**Soluble adhesion-molecules and leukocyte expression of adhesion molecules** Several authors have reported elevated levels of soluble (s) VCAM-1, sICAM-1 and sE-selectin (Gruschwitz 1995, Denton 1995, Ihn 1997, Stratton 1998, Sondergaard 1998) and the finding of correlations between circulating levels and signs of disease activity speak in favour of a pathogenetic role (Denton 1995, Ihn 1998). Accordingly, the highest circulating levels of sICAM-1, sVCAM-1, sP-selectin and to a lesser degree sE-selectin are found in SSc patients with early, active, oedematous skin lesion, and correlate to dermal endothelial cell adhesion molecule expression (Gruschwitz 1995, 1997).
Figure 3
ENDOTHELIAL ADHESION MOLECULES IN LEUKOCYTE MIGRATION

1. Rolling  
2. Arrest  
3. Migration

Model of leukocyte adhesion and transmigration. Rolling of the leukocytes is mediated by molecules of the selectin family. Firm attachment is mediated by the interaction of the integrins LFA-1 and VLA-4 on leukocytes with adhesion molecules of the immunoglobulin family (VCAM-1 and ICAM-1). Transmigration requires a chemoattractant gradient and the interaction of platelet/endothelial adhesion (PECAM-1) with PECAM-1 on the leukocyte.

E-selectin = endothelial selectin, ESL-1 = E-selectin ligand 1, ICAM-1 = intercellular adhesion molecule 1, LFA-1 = leukocyte function-related antigen 1, LSL = L-selectin ligand, PSGL-1 = p-selectin glycoprotein ligand 1, VCAM-1 = vascular adhesion molecule 1, VLA-1 = very late activation antigen 1.

From Behrendt 2002. Am J Cardiol;90(suppl):40L-48L

Furthermore, soluble adhesion molecule levels may mirror SRC, while findings in IPHT in SSc diverge (Denton 1995, Stratton 1998).

The differential expression of ligands by peripheral blood leukocytes may indicate the organ targeted. Thus increased expressing of the α4β1 integrin in circulating Th cells, tropic to VCAM-1 on endothelial cells in bronchus related lymphoid tissue (BALT), may correlate to severity of ILD in SSc. Moreover, the number of α4β7 integrin bearing Th cells committed to the mucosal vascular adhesion molecule MadCAM is especially high in dSSc with oesophageal involvement (Scala 2005). Activated Th cells in SSc express the integrins: leucocyte function-related antigen 1 (LFA-1), (the ligand of ICAM-1), very late activation antigen 4 (VLA-4), (the ligand of VCAM-1), homing CAM (H-CAM) and ICAM-1 and have an increased capacity for transendothelial diapedesis, which may potentially lead to organ damage (Stummvoll 2004).

Adhesion-molecules in the skin lesion In SSc lesional skin, increased expression of ICAM-1, VCAM-1, E-selectin and P-selectin seems to play an important role in the homing of activated T lymphocytes, especially Th cells. ICAM-1 is expressed on several cell types including endothelial cells (Gruschwitz 1995), fibroblasts (Sollberg 1992), perivascular Th cells and monocytes (Sondergaard 1998). Even the β1 subunit of the integrin ligand for ICAM-1 is present on perivascular lymphoid cell infiltrates (Sollberg 1992). TNFα and IFNγ (Gruschwitz 1997, Shi-Wen 1994) present in the SSc skin lesion may upregulate ICAM-1 expression on various cell types. VCAM-1 has been found to be more exclusively expressed on skin endothelial cells than ICAM-1, while E-selectin is expressed exclusively by endothelial cells (Sollberg 1992, Gruschwitz 1995).

Adhesion-molecules in interstitial lung disease Studies are few on adhesion molecule expression in SSc lung. Alveolar lining cells express ICAM-1 constitutively and may increase the expression in idiopathic ILD in regions characterized by septal hypertrophy and cellular infiltration as well as in regions with honeycombing (Nakao 1995). Endothelial cells in small lung vessels may show weak expression of ICAM-1constitutively, while in areas with alveolitis as well as with honeycombing in idiopathic ILD, endothelial cells on small vessels and capillaries almost universally express ICAM-1. Patchy expression of VCAM-1 and E-selectin around blood vessels has been found in ILD in SSc as well as in normal lung. In broncho-alveolar lavage fluid (BALF) from SSc patients with ILD, sE-selectin is detected more often and is associated with BALF lymphocytosis (Southcott 1998). It has been speculated that increase in E-selectin expression and shedding might be associated with the migration
of neutrophils into regions with honeycombing (Nakao 1995), -with increased interstitial lymphocyte accumulation and with endothelial leakage (Southcott 1998).

**NITRIC OXIDE IN SYSTEMIC SCLEROSIS**

**Introduction** NO is synthesized from arginine (Palmer 1987) by three isoforms of nitric oxide synthase (NOS): endothelial NOS (eNOS, NOS 3); inducible NOS (iNOS, NOS 2) and neuronal NOS (nNOS, NOS 1). The NOSs are complex haemoproteins, which contain both oxidative and reductive domains. NOS activity requires a number of cofactors, such as tightly bound flavoproteins and tetrahydrobiopterin (BH4), while the production of NO itself requires the co-substrate oxygen (O₂) and nicotinamide adenine dinucleotide phosphate (NADPH). The reductase domain of NOS provides electrons from NADPH to the haem domain, where oxidation of L-arginine to NO and citrulline occurs. Endothelial NOS is a low-output, Ca²⁺ dependent enzyme, which produces NO in a pulsatile manner and is expressed constitutively in endothelial cells. NO produced by eNOS is responsible for basal vascular tone homeostasis (Michel 1992).

![Diagram of the eNOS signaling pathway](image)

**Figure 4** THE eNOS SIGNALING PATHWAY

BH₄ = tetrahydrobiopterin 4, cGMP = cyclic guanosine monophosphate, eNOS = endothelial nitric oxide synthase, GC = guanylate cyclase, GTP = guanosine triphosphate, NADPH = reduced nicotinamide-adenine dinucleotide phosphate, NO = nitric oxide.

*From Behrendt 2002. Am J Cardiol;90(suppl):40L-48L*

On the opposite, iNOS is a high-output enzyme, which produces NO continuously until degradation. The amount of iNOS is increased drastically upon cell stimulation by pro-inflammatory cytokines such as TNF-α, IL-1 and IL-6 (Lyons 1992) via activation of the transcription factor NFκB -and by IFNγ via several mechanisms among others the activation of the JAK2/STAT1 pathway (Ganster 2001). Hypoxia may induce iNOS via involvement of CCAAT-enhanced box binding protein (C/EBP) (Teng 2002). The elaborate composition of the human iNOS promoter with several binding sites for NFκB, STAT1 and other activation and repression factors assures controlled iNOS production. The induction of iNOS via NFκB is inhibited by glucocorticoids (Kunz 1994) and TGFβ (Gilbert 1993) and may also be blocked by cytokines of the Th2 cell subset (Paludan 1999). Moreover, iNOS induction may be influenced by negative- and positive feed-back mechanisms involving NO, cGMP and peroxynitrite (ONOO⁻) (Peng 1998, Cooke 2002). Inducible NOS has been found in a variety of cells: endothelial cells, VSMC, macrophages, neutrophils and fibroblasts. Endothelially produced NO diffuses into adjacent VSMCs activating guanylate cyclase to increase cGMP levels, resulting in VSMC relaxation and subsequent vasodilation. The greatest part of endothelial NO may, however, diffuse into the bloodstream and into the erythrocyte, where it is momentarily oxidised by oxyhemoglobin to nitrite (NO₂⁻) and nitrate (NO₃⁻), of which the latter is the major metabolite of NO in the circulation (Wennmalm 1993) (figure 5). NO may react with proteins to form nitrosoproteins.
**Figure 5**  THE METABOLISM OF NITRIC OXIDE IN VIVO

**NO** is generated from L-arginine in endothelial cells (EC) and diffuses to the vascular lumen, from where it is taken up in the erythrocyte (RBC) or abluminally to the vascular smooth muscle cell (VSMC). In the erythrocyte, NO may react with oxyhemoglobin (HbO$_2$) to form methemoglobin (HbFe$^{3+}$) and nitrate (NO$_3^-$) or with hemoglobin to form nitrosohemoglobin (HbNO). HbNO may then when the oxygen pressure increases be converted to HbFe$^{3+}$ and NO$_3^-$. If NO is converted to nitrite (NO$_2^-$) before being taken up in the erythrocyte, the same products are formed, that is HbNO, NO$_3^-$ and MetHb. Nitrate (NO$_3^-$) diffuses to plasma and is cleared via the kidneys.  


**Circulating nitric oxide metabolites** Most studies on circulating levels of degradation products of NO or surrogate markers of NO production, such as nitrated proteins and citrulline, speak in favour of an increased NO synthesis in SSc. In an early report, levels of NO metabolites: nitrite and nitrate were elevated in lSSc as well as in dSSc (Yamamoto 1998). These observations are corroborated by the findings by Sud et al of increased serum nitrite -and citrulline levels in both ISSc and dSSc (Sud 2000). Increased NO synthesis may mirror an active disease process in SSc, as the sum of serum NO metabolites is highest in patients with early, extensive cutaneous engagement and in patients with active ILD based on the finding of ground-glass appearance on high resolution computed tomography (HRCT) of the lungs (Takagi 2003). Others have found NO metabolite levels increased in SSc without pulmonary hypertension, but normal in SSc with IPHT (Kawaguchi 2006). It is controversial whether peripheral blood mononuclear cells (PBMC) from SSc patients produce increased amounts of NO (Yamamoto 1998, Allanore 2001).

In favour of decreased serum nitrate levels in SSc speak an early report (Kahaleh 1993) and the finding of decreased iNOS expression in PBMC spontaneously as well as after stimulation with pro-inflammatory cytokines and IL-4 (Allanore 2001). The discrepancies may be explained by differences in patient characteristics and disease activity, thus blood NO metabolite levels in ISSc- and late stage SSc without ILD may not differ from levels in normal controls (Takagi 2003).

**Nitric oxide synthases in the skin lesion** According to the result of a differentiated evaluation of the endothelial expression of the eNOS and the iNOS proteins in different stages of the SSc skin lesion, eNOS predominates in endothelial cells in normal and uninvolved SSc skin, while iNOS achieves an increasingly dominating role as the skin lesion evolves. Moreover, SSc endothelial cells harbour nitrotyrosine, as a marker of free radical damage to cellular proteins by NO or peroxynitrite (ONOO$^-$), which is a by product of NO formation (Cotton 1999). The theory proposed lately that eNOS and iNOS are inversely regulated may thus apply to the advancing oxidant changes in SSc skin lesions (Persichini 2006). This suggestion is further supported by the finding of a relative deficiency of eNOS in dermal microvascular endothelial cells from SSc patients (Romero 2000). By means of immuno-histochemical...
staining the question as to the nature of the cells and enzymes engaged in NO production in SSc skin lesions has been explored. Also mononuclear infiltrating cells and skin fibroblasts express the iNOS enzyme and cultured fibroblasts from the same skin lesion express the iNOS gene (Yamamoto 1998) as well as the enzyme and in contrast to normal skin fibroblasts produce NO spontaneously (Takagi 2003).

**Nitric oxide and lung disease** Levels of exhaled NO are increased in SSc with ILD, especially in patients with high neutrophil-, lymphocyte- or eosinophil percentage in BALF (Paredi 1999, Rolla 2000). In recent studies, using a refined method, alveolar NO concentration was shown elevated in ILD, correlating to extension of ground glass changes and to reticular score (Girgis 2002, Tiev 2007). Reports on exhaled NO levels in SSc patients with PHT are contradictory. Thus exhaled NO both in patients with combined ILD and PHT and in IPHT was found reduced by some authors (Kharitonov 1997, Rolla 1998, 2000), unaltered (Girgis 2002) or elevated (Tiev 2007).

The increased NO synthesis in ILD in SSc may take place in macrophages, neutrophils and alveolar epithelium, as iNOS expression has been found upregulated in these cells in idiopathic pulmonary fibrosis (Saleh 1997). It is possible that high levels of NO within the alveolar space may perpetuate the inflammatory and fibrotic process and participate in vascular injury. In patients with the idiopathic form of ILD, corticosteroids lower exhaled NO levels (Paredi 1999), a finding in agreement with the known inhibiting effect of glucocorticoids on iNOS (Radomski 1990). Measurement of exhaled NO has been proposed as a non-invasive tool to follow inflammatory activity in ILD in SSc.

**Nitric oxide and the proliferation of endothelial cells** Although not confirmed, it seems reasonable to assume that NO plays a role in the development of the typical vascular lesions in SSc. Increased NO formation may lead to altered angiogenesis via the induction of the synthesis of the vascular endothelial growth factor (VEGF). Experimentally, the transfection of rat VSMC with the eNOS gene results in increased VEGF synthesis, which promotes angiogenesis, endothelial cell- proliferation, survival and migration (Dulak 2000). In SSc, the role of increased levels of VEGF is debated as levels are high in dSSc patients without fingertip ulcers (Distler 2002) but also correlate inversely with nailfold capillary density (Choi 2003).

**Nitric oxide dependent mechanisms in the therapy of systemic sclerosis** (figure 6) Agents with impact on the NO pathway are effective therapeutics in SSc vascular disease. NO inhalation significantly decreases pulmonary arterial pressure (Hoep 2000), but is hampered by extremely short half-life, tendency to rebound effect upon interruption and tolerance development (Sitbon 1995, Williamsson 1996, Miller 1995). Cyclic GMP produced by the action of NO on VSMC soluble guanylate cyclase and of atrial natriuretic protein (ANP) and brain (B) NP on VSMC membrane bound GC receptors is hydrolysed to GMP by phosphodiesterase (PDE) 5. Thus the inhibition of PDE-5 leads to an increase in cGMP, increased relaxation of VSMCs and vasodilation. The synthetic phosphodiesterase inhibitor sildenafil inhibits PDE-5 and thus augments the actions of NO. Several studies point at a beneficial effect of sildenafil on endothelial function with increase in endothelial dependent dilation and decrease in serum endothelin and von Willebrand factor (Rossi 2008).

In SSc, Sildenafil may have beneficial effects on duration, frequency and severity of Raynaud’s phenomenon with increase in capillary blood-flow velocity (Fries 2005). Sildenafil may also have beneficial effects in PHT secondary to connective tissue disease, increasing cardiac output and 6 minute walk test (Galie 2005) and decreasing right ventricular mass and lower BNP, which is a measure of cardiac strain (Wilkins 2005).
SYNTHESIS AND BREAK-DOWN OF CGMP

Synthesis and breakdown of cyclic guanosine monophosphate (cGMP) and action of phosphodiesterase 5 (PDE-5) inhibitors sildenafil, vardenafil and tadalafil.

ATP = adenosine triphosphate; GTP = guanosine triphosphate; cAMP = cyclic adenosine monophosphate; AMP = adenosine monophosphate; GMP = guanosine monophosphate.


INTERSTITIAL LUNG DISEASE IN SYSTEMIC SCLEROSIS

Epidemiology In an early autopsy study 75% of 58 consecutive SSc cases showed lung fibrosis often symmetrically located to the lower lobes (D’Angelo 1969). In a later study 27% of 890 SSc patients developed moderate restrictive lung disease with forced vital capacity (FVC) 50-75%, while 13% developed severe restrictive lung disease with FVC < 50%. Predisposed are males with early, diffuse skin engagement (Steen 1994). In patients with severe disease median survival from first disease symptom was 9.8 years and from first lung symptom 5.3 years, 40% died from lung disease and in 27% lung disease was a major contributing cause of death (Steen 1994). Advanced fibrosis with honeycombing in all lung segments may be present in 50% of dSSc patients and in 16% of lSSc patients, in whom a light fibrosis of the lower lobes is however more common (23%) (Devenyi 1995). It is controversial whether Scl-70 antibodies are more frequently found in SSc with ILD (Steen 1994, Greidinger 1998).

High resolution computed tomography HRCT with thin sections of 1.5-3 mm may reveal early and/or minor interstitial lung changes not detectable by plain X-ray (Devenyi 1995). Lung parenchyma changes depicted by HRCT are of 5 types: 1) ground glass change, which is a hazy increased density of the lung parenchyma, believed to signify alveolar and interstitial accumulation of inflammatory cells, 2) septal/subpleural lines, which are thickening of the interlobular interstitium by oedema or fibrosis, 3) irregular pleural margins, which denote fibrotic changes at the pleural-parenchymal interface, 4) reticulate pattern fibrosis and 5) honeycombing, which equalizes end-stage fibrosis with parenchymal destruction (Warrick 1991). HRCT may be used to estimate lung function and inflammatory status as severity of HRCT changes correlate to functional tests such as DLCO (Warrick 1991), vital capacity (VC), bronchoalveolar lavage fluid (BALF) cellularity (Remy-Jardin 1993) and clearance of inhaled 99mTc-DTPA aerosol (Pignone 1992).

Bronchoalveolar lavage fluid cell count: relation to HRCT and lung function In ILD in SSc, inflammatory cells are recruited to the alveolar spaces. To diagnose and/or investigate ILD, cells and alveolar lining fluid are sampled by BAL from one lobe, -often the middle-, which is considered
Neutrophil alveolitis: relation to lung function, HRCT and prognosis

Neutrophil alveolitis has been defined slightly differently in various studies: e.g. neutrophil percentage 2 SD above the mean value in healthy controls (Silver 1984, Edelson 1985, Steen 1994), neutrophil percentage above 4% (Wallaert 1986, Bouros 2002) or 5% as recommended by the American Thoracic Society (Behr 1996), or as high as 10% (Konig 1984).

Neutrophil alveolitis is associated with abnormal lung function, that is signs of restrictive lung disease (Wallaert 1986, Silver 1990, Remy-Jardin 1993) and predicts accelerated decline in lung volumes (Silver 1990, Behr 1996) and in DLCO (Konig 1984, Silver 1990, Behr 1996) and can not be ascribed to bacterial infections, smoking habits (Wallaert 1986) or oesophageal involvement (Edelson 1985). It has been discussed, whether neutrophil alveolitis is an early sign correlated to ground glass changes or a late sign correlated to fibrotic changes on HRCT. In a comparative study, all patients with severe ground glass changes and diffuse honeycombing (often found in combination) had neutrophil alveolitis (Remy-Jardin 1993). Others have observed high neutrophil count in patients with extensive reticulate pattern fibrosis, suggesting neutrophil influx predominantly in advanced lung disease (Wells 1994) as opposed to reported higher neutrophil count in patients with ground glass changes and lung symptoms of short duration (Warrick 1991, Owens 1986). However, neutrophil alveolitis may also be present in patients without lung symptoms and with a normal chest radiography (Wallaert 1986) and normal HRCT (Remy-Jardin 1993). The correlation found between neutrophil percentage and fall in lung function index identifies neutrophil alveolitis as important for outcome. In line with this finding, patients with active BALF deteriorated more when left untreated and improved more when treated with CYC than patients with an inactive BALF (Behr 1996).

Immune mechanisms in neutrophil alveolitis

In the process of ILD development, mononuclear cells may be activated early and secrete neutrophil homing chemokines. IL-8, a powerful neutrophil attractant and activator is secreted at increased levels by macrophages in SSc ILD (Crestani 1994). Moreover, elevated BALF IL-8 levels and presence of transcripts in the BALF cell pellet correlate to neutrophil percentage (Southcott 1995). Neutrophils may release matrix metalloproteinase-9 (MMP-9), which potentiates IL-8 effects a ten-fold through truncation of the cytokine. Truncated IL-8 effectively stimulates immediate MMP-9 release and production, achieving a positive spiral for neutrophil homing. Growth related oncogene-alpha (GRO-α) and epithelial neutrophil-activating protein 78 (ENA-78) like IL-8 are CXC chemokines with neutrophil attractant properties. The synthesis and release of all three chemokines may be induced in alveolar epithelial cells, monocytes and endothelial cells by IL-1β, TNFα and Th2 cytokines: IL-4 and IL-13 (Meyer-Hoffert 2003) via NFKB and mitogen activated protein kinase (MAPK) transcription pathways (Issa 2006). Dexamethasone and IFNγ downregulate their synthesis. GRO-α and IL-8 may even delay neutrophil apoptosis (Glynn 2002). Also the activity of ENA-78 may be increased through truncation by MMP-9.

There are no reports on levels of GRO-α and ENA-78 in SSc BALF, however, elevated serum IL-8 and GRO-α levels correlate with decrease in DLCO and VC in SSc (Furuse 2003). In
idiopathic ILD blood neutrophils seem primed by IL-8 and GRO-α to readily change shape and exert increased motility (Glynn 2002), while in BALF, ENA-78 level is elevated and correlates with number of neutrophils (Nakayama 2005). In patients with chronic obstructive pulmonary disease (COPD) primary bronchial epithelial cell GRO-α transcription and GRO-α protein release is increased (Schulz 2004).

**Neutrophil proteases**

**Introduction** Various inflammatory cell products contribute to the disruption and fibrosis of the alveolar interstitium in ILD in SSC, especially the balance between proteases and anti-proteases decides outcome. Neutrophils may be important because of their capacity to secrete various proteases, which modulate collagen synthesis and break-down. The proteases are contained in granules named after the time of their appearance during neutrophil maturation. Primary azurophilic granules containing myeloperoxidase (MPO), neutrophil elastase, cathepsin B, proteinase 3 and α1-antitrypsin, appear early during the myeloblast stage, undergo limited exocytosis and contribute primarily to the killing and degradation of engulfed micro-organisms in the phagosomes. MPO negative granules are divided into specific (secondary) granules and MMP-9 containing (tertiary) granules. Tertiary granules function as a reservoir of matrix degrading enzymes and are exocytosed to aid in extravasation and diapedesis.

**Myeloperoxidase (MPO)** In neutrophil phagosomes molecular oxygen (O₂) is reduced to superoxide anion (O₂⁻) by electrons pumped from cytosol NADPH by phagocyte NADPH oxidase (phox). This charge transfer is compensated by the influx of protons (H⁺) into the phagosome, which are used to reduce superoxide anion (O₂⁻) to hydrogen peroxide (H₂O₂). MPO catalyses the reaction between H₂O₂ and a halide, producing for example hypochlorous acid (HOCI) (household bleach). Hypochlorous acid is far more toxic to bacteria and cells than H₂O₂ and may in turn react to form secondary chloramines, which are much more stable, but almost as noxious as HOCl. In 1993, Shanez et al failed to show raised BALF MPO levels in ILD in SSc by means of a RIA method and so did Cailes et al in 1996, who used a biological assay (Shanez 1993, Cailes 1996). However, Cailes et al showed increased BALF MPO levels in idiopathic ILD, in which there is a higher neutrophil percentage (Cailes 1996) and in which levels of MPO correlate with decrease in lung function (Hällgren 1989).

**Neutrophil elastase** Neutrophil elastase (NE), which is a serine protease, constitutes the major part of the primary granules and is a potent proteolytic agent capable of degrading extracellular matrix (ECM) proteins including elastin, but also clotting factors and the tissue inhibitors of matrix metalloproteinases (TIMPs). Moreover, NE may induce IL-8 production in epithelial cells and thus promote further neutrophil influx and MMP-9 release (Nakamura 1992, Kuwahara 2006). Deficiency of α1-antitrypsin, which is the main anti-protease of NE, leads to lung tissue degradation. In BALF from SSC patients with ILD increased amounts of unbound and α1 antitrypsin complexed NE correlate to neutrophil percentage and decline in lung function (Crestani 1994, Cailes 1996).

**MMP-9 (92 kDa gelatinase, neutrophil gelatinase B or 92 kDa type 4 collagenase)** In the neutrophil, MMP-9 is the predominant metalloproteinase synthesized and is stored in the tertiary granules together with MMP-8, MMP-25, lipocalin and lactoferrin. Recently, it has been appreciated that MMP-9 apart from functioning as an effector molecule in innate immunity also is an important regulator of the adaptive immune system and a potential target for inhibition in various diseases (Opdenakker 2001, review).

Matrix metalloproteinases (MMPs) are a family of about 25 zinc- and calcium dependent endopeptidases with distinct substrate specificities so that they can degrade most components of the ECM. The MMPs play an important role in the physiologic and pathologic processes of ECM homeostasis and turnover, tissue degradation and repair, cell migration and inflammation. Three types of MMPs have been identified: the gelatinases, collagenases and stromelysins, all differing as to tissue expression and substrate specificity. Neutrophils, eosinophils, macrophages and epithelial cells are major sources of MMPs. MMP activity is physiologically controlled by a class of anti-proteases, the tissue inhibitors of metalloproteinases (TIMPs).

The secreted inactive zymogen pro-MMP-9 contains a pro-peptide that interacts with the zinc ion in the active site. Removal of the pro-peptide by enzymes like serine proteases or other MMPs (MMP-2, MMP-3, MMP-7) (Nagase 1996, Ogata 1992) enables the zinc ion to engage in an essential interaction with a water molecule, rendering the enzyme into an 84-kDa active form. TIMP-1 binds reversibly to the zinc binding catalytic site of active MMP-9 and to the hemopexin moiety of pro-MMP-9. TIMP-1 is the TIMP found in highest concentrations, binds to MMPs in a 1:1 molar ratio and inhibits gelatinase-type MMPs: MMP-9 and MMP-2 (gelatinase A) as well as MMP-1. TIMP-1 also inhibits
stromeolysin-type MMPs, of which MMP-3 is involved in the activation of MMP-9. TIMP-1 is secreted in association with MMP-9 by several cell types, see below.

MMP-9 may degrade native collagens IV and V, denatured collagens (gelatins), proteoglycans and elastin and is produced in large amounts constitutively -in free form- by neutrophils (Sopata 1974). Furthermore, MMP-9 may be produced free and TIMP-1 complexed by eosinophils (Okada 1997), mast cells (Baram 2001), macrophages/monocytes (Mainardi 1984), lymphocytes, NK cells, dendritic cells and epithelial cells (Hozumi 2001) upon induction with pro-inflammatory cytokines (Opdenakker 1994), basement membrane, adhesion molecules and complement fragments. Invading cells, stimulated by contact with basement membrane, produce MMP-9, to facilitate migration.

In the basement membrane of the alveolar wall, collagen IV occupies a substantial portion and is of critical importance to structural integrity. Moreover, elastin comprises about 2.5% of the dry weight of the lung and is vital to the normal structure and function (Starcher 1986, Shapiro 1991). In the small airways, elastin maintains patency and alveolar wall resilience. In the pathogenesis of idiopathic pulmonary fibrosis, disruption of the epithelial basement membrane is known to be associated with fibroblast migration into the alveolar spaces and is considered a key event leading to intraluminal fibrosis.

Stimulation and suppression of MMP-9 production are complex and differ between cell types. In general Th1 pro-inflammatory cytokines such as TNFα and IL-1β via a NFKB mediated pathway, enhance MMP-9 production in monocytes/macrophages, dendritic cells and epithelial cells (Zhang 1998, Hozumi 2001, Mautino 1999), glucocorticoids (Shapiro 1991), TGFβ1 (Kerr 1990) and Th2 cytokines: IL-4 and IL-10 may block MMP-9 synthesis and enhance TIMP-1 production (Lacraz 1992, 1995). Lately mast cells have been shown to increase neutrophil production and release of MMP-9 via tryptase (Vliagoftis 2000) and probably even histamine activated pathways (Shimizu 2006).

In the neutrophil, MMP-9 is stored in the tertiary granules and secreted in its inactive, uncomplexed pro-form. The pro-form may be activated by MMP-3 (stromeolysin) and MMP-7 (matrilysin). The serine proteases: tryptase and chymase, released by mast cells, may activate MMP-3, which in turn may activate MMP-9 (Lees 1994); chymase even has the ability to activate MMP-9 directly (DiScipio 2006). Furthermore, plasminogen activator, cathepsin, HCl, reactive oxygen species, and others may activate MMP-9.

Beyond the ability to degrade ECM, MMP-9 functions to regulate neutrophil biology (Opdenakker 2001) through positive feedback mechanisms involving interaction with IL-8 and IL-1β. Beyond truncating IL-8 and ENA-78, MMP-9 may cleave pro IL-1β to IL-1β, and thus establish another positive feedback loop as this cytokine may enhance/induce MMP-9 secretion in several cell types including the eosinophil, lymphocytes and macrophages. The cleaving of ECM proteins may furthermore result in the formation of neo-epitopes, so-called remnant epitopes that generate autoimmunity (REGA model), activating new specific Th cells to participate in the auto-immune self-perpetuating reaction thought to cause ILD in SSc (Van den Steen 2002) (table 6).

### Table 6.
**MMP-9 substrates and immune functions**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Effects on immune function</th>
<th>References</th>
</tr>
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<tbody>
<tr>
<td>IL-8</td>
<td>Potentiates 10 fold through aminoterminal truncation</td>
<td>Van den Steen 2000</td>
</tr>
<tr>
<td>ENA-78</td>
<td>Early potentiation, late inactivation through aminoterminal truncation</td>
<td>Van den Steen 2003</td>
</tr>
<tr>
<td>Pro IL-1β</td>
<td>Activation</td>
<td>Schönbeck 1998</td>
</tr>
<tr>
<td>GRO-α</td>
<td>Degradation/inactivation</td>
<td>Van den Steen 2000</td>
</tr>
<tr>
<td>IL-2 Rα (CD25)</td>
<td>Inactivation</td>
<td>Sheu 2001</td>
</tr>
<tr>
<td>Type II collagen</td>
<td>REGA model in RA</td>
<td>Van den Steen 2002</td>
</tr>
<tr>
<td>Myelin basic protein</td>
<td>REGA model in MS</td>
<td>Proost 1993</td>
</tr>
</tbody>
</table>

IL-8 = interleukin 8, ENA-78 = epithelial neutrophil activating protein 78. GRO-α = growth related oncogene alpha, IL-2 Rα = interleukin 2 receptor alpha chain, REGA = remnant epitopes, that generate autoimmunity.

Increased MMP-9 production has been found in several lung diseases, characterised by the degradation and remodelling of lung interstitial tissue and airways such as idiopathic lung fibrosis (Suga 2000, Beeh 2003), emphysema (Finlay 1997), chronic obstructive lung disease (COPD) (Segura-Valdez 2000, Russell 2002), the acute respiratory distress syndrome (ARDS) (Ricou 1996, Torii 1997) and in asthma (Mautino 1999, Cataldo 2004).
In MMP-9 knock-out mice, MMP-9 has been shown essential for the migration of stem cells into the blood stream and to the peripherisation of leukocytes to sites of inflammation (D’Haese 2000). Moreover, these mouse strains seem protected against certain autoimmune diseases (Liu 1998).

**MMP-9 inhibitors**

Several reasons to believe that selective MMP-9 inhibitors (MMPI) may reduce the harmful effects of ILD in SSc. Thus in bleomycin induced experimental lung fibrosis, MMP-9 knock-out mice develop less alveolar bronchiolization than wildtype (Betsuakui 2000). D-penicillamine and modified tetracyclines such as minocycline (Yrjanheikki 1999), inhibit MMPs by their ability to bind metal ions. The substrate analogue, small molecule inhibitors, to which batimastat belongs, bind to the zink ion and reduce MMP-2 and -9 levels in BALF as well as lung hydroxyproline content and diminish histopathological fibrotic changes in experimental ILD (Corbel 2001). Other types of MMPIs to come are synthetic TIMPs, monoclonal antibodies against MMPs and “suicide” MMPIs, which bind to the active site zink and cause conformational distortion (Rosenblum 2003).

**Eosinophils in interstitial lung disease**

Eosinophils gather in the alveolar interstitium in ILD in SSc and some authors have used BALF eosinophil percentage as a hallmark to define ILD. Some have chosen an eosinophil percentage higher than 2 SD above the mean in healthy subjects (Silver 1984, Owens 1986) others eosinophil percentage above 2% (Gustafsson 1991, Remy-Jardin 1993), while some have set the limit as high as 4% (Wallaert 1986) to denote ILD, in which a high BALF eosinophil percentage may predict a more progressive course (Silver 1993, Atamas 1999).

In SSc, eosinophils accumulate in lung, skin and gut (Gustafsson 1991, Cox 1995, DeSchryver-Kecskeméti 1989). The trafficking of eosinophils into inflamed tissues in particular involves Th2 cytokines: IL-4, 5 and 13, endothelial adhesion molecules, and chemokines (RANTES, eotaxin), where IL-5 and eotaxin selectively regulate eosinophil homing (Collins 1995, Zimmerman 2003). Eosinophils are themselves an important source of Th2 cytokines IL-4 and 5 (Justice 2002). While IL-5 stimulates activation, differentiation and survival, IL-4 and 13 induce eotaxin synthesis in tissue-resident as well as in infiltrating cells. Furthermore, eosinophils may communicate with T-cells, serving as antigen presenting cells (APCs) and forwarding Th2 polarisation through the synthesis of IL-4, 6, 10 and 13 (Rumbley 1999, Voehringer 2004, Mattes 2002). In rapidly progressive ILD in SSc, alveolar CD8+ T cells synthesise increased amounts of IL-5 and IL-4 and eosinophil number is greater than in patients without IL-4 and 5 production (Atamas 1999), which may explain the alveolar eosinophilia and elevated BALF levels of eosinophilic cationic protein (ECP) and major basic protein (MBP) found in SSc (Gustafsson 1991, Cox 1995).

The eosinophil may damage tissues through the release of toxic basic proteins from their granules (Hermas 1992, Mitra 2000, Abu-Ghazaleh 1992) and through the release of neutrophil elastase and MMP-9 during migration (Lungarella 1992, Okada 1997). The proteins, including ECP, MBP and eosinophil peroxidase (EPO) are potent toxins to helminths, bacteria, protozoae and mammalian cells. ECP has gene homology to pancreatic ribonuclease and constitutes about one third of total mass eosinophil granule protein (Olsson 1977). MBP has an ability to adhere to cellular- and ECM debris and is believed to function as a “tag” to recruit scavenger cells (Moy 1990), thus forwarding tissue remodelling (Temkin 2004). EPO catalyses the reaction between hydrogenperoxide and NO to form peroxynitrile, promoting cell necrosis and apoptosis.

Eosinophils and mast cells colocalize in inflamed tissue and may communicate as both MBP and ECP cause mast cell degranulation (Shakoory 2004) and release of mast cell chymase, which in turn may cause eosinophil release of mast cell growth factor: stem cell factor (SCF) (Hartman 2001). In an early study, ECP levels were high in SSc sera, BALF and skin, BALF ECP correlating to decrease in lung function (Gustafsson 1991). MBP is also present in SSc skin and lung tissue and increased levels of circulating MBP correlate with the extent of cutaneous involvement and lung restrictivity (Cox 1995). In the eosinophil, MMP-9 is stored in granules, which are much fewer than in the neutrophil, but during the oxidative cell burst –of which even the eosinophil is capable- EPO, MPO and NO are released, which may all activate MMP-9 (Gu 2002, diScipio 2006).

**Mast cells in interstitial lung disease**

In SSc, mast cells are found in increased number almost ubiquitously, i.e. in skin, blood, lungs, myocardium and gastric wall (Hawkins 1985, Seibold 1990, Irani 1992, Chanez 1993, Lichtbruna 1990, Ibbav-Manneschi 2002) reflected in elevated serum levels of tryptase and histamine, especially in patients with rapidly progressive skin involvement (Irani 1992, Falanga 1990). Mast cell homing, activation and proliferation are stimulated by Th2 cytokines (IL-4, 5, 6, 10), stem cell factor (SCF) (c-kit ligand) secreted mainly by fibroblasts, and contact with IgE (Austen 2001, review).

In ILD in SSc, alveolar mast cell number is increased (Shanez 1993, Behr 1996) and lung tissue mast cells activated, expressing the c-Kit receptor for SCF and the FcɛRI for IgE (Edwards 2005). Activated mast cells tend to degranulate and release preformed inflammatory mediators,
including histamine, cytokines such as TNFα, IL-4, IL-6, TGFβ (Baram 2001), metalloproteinases: MMP-9, MMP-3 and their inhibitor: TIMP-1 and specific serine proteases: trypAmes and chymes, involved in MMP-9 activation (Tanaka 1999).

In SSC, mast cells accumulate in clinically involved- and uninvolved lung tissue (Seibold 1990, Chanez 1993) and alveolar spaces, independent of HRCT changes (Behr 1996). Therefore, the mast cell may be an early active cell in ILD in SSC (Chanez 1993), as it is often situated in proximity to fibroblasts (Harrison 1991, Majumdar 1999) where it may exert fibrogenic effects through the secretion of fibroblast growth factors: TGFβ, basic fibroblast growth factor (bFGF) and platelet derived growth factor (PDGF) (Lindstedt 2001, Li 2002). Moreover, trypMase (Cairns 1997, Gruber 1997), histamine (Hatamochi 1985, Garbuzenko 2002, 2004) as well as TIMP-1 (Kikuchi 1997) may stimulate fibroblast proliferation and collagen production. In ILD in SSC alveolar levels of trypMase and histamine are high (Chanez 1993).

<table>
<thead>
<tr>
<th>First Author</th>
<th>Enzyme involved</th>
<th>Mechanism</th>
<th>Enzyme secreting cell</th>
<th>Comment</th>
</tr>
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<tbody>
<tr>
<td>Nagase 1990</td>
<td>Neutrophil elastase</td>
<td>Activates pro-MMP-3</td>
<td>Neutrophil eosinophil</td>
<td>MMP-3 in turn activates MMP-9</td>
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<td>Ogata 1992</td>
<td>MMP-3</td>
<td>Activates pro-MMP-9</td>
<td>Fibroblast, macrophage</td>
<td>MMP-3 from sedentary cells may activate MMP-9 from invading cells</td>
</tr>
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<td>Lees 1994</td>
<td>Chymase</td>
<td>Activates pro-MMP-3, and -1</td>
<td>Mast cell</td>
<td>Does not activate pro-MMP-2, 9 directly</td>
</tr>
<tr>
<td>Lees 1994</td>
<td>Tryptase</td>
<td>Activates pro-MMP-3</td>
<td>Mast cell</td>
<td>Does not activate pro-MMP-1, -2, -9 directly</td>
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<tr>
<td>Itoh 1995</td>
<td>Neutrophil elastase</td>
<td>Inactivates TIMP-1 bound to MMP-9</td>
<td>Neutrophil eosinophil</td>
<td>Renders pro-MMP-9 activateable to MMP-3</td>
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<td>Fang 1996</td>
<td>Chymase</td>
<td>Activates pro-MMP-9</td>
<td>Mast cell</td>
<td></td>
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<td>Frank 2001</td>
<td>Chymase</td>
<td>Inactivates unbound and MMP-9 complexed TIMP-1</td>
<td>Mast cell</td>
<td>Chymase activates TIMP-1 complexed, as well as free pro-MMP-9,</td>
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<tr>
<td>Tchougounova 2005</td>
<td>Chymase</td>
<td>Activates MMP-9, and -2</td>
<td>Mast cell</td>
<td>Chymase deficient mice lack active MMP-9 and show increased fibronectin content in the lung.</td>
</tr>
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<td>Shimizu 2006</td>
<td>Histamine</td>
<td>May increase MMP-9 and -2 production in neutrophils</td>
<td>Mast cell</td>
<td>Anti-histamines may decrease MMP-9 production in neutrophils</td>
</tr>
</tbody>
</table>

MMP-3 = metalloproteinase 3 (stromelysin); TIMP-1 = tissue inhibitor of metalloproteinase 1

Mast cells de novo produce and release MMP-9 upon contact with activated T-cells and/or binding to SCF synthesized by fibroblasts, an effect opposed by TGFβ, (Fang 1999). Mast cells in bronchial subepithelial tissue produce and harbour MMP-9 (Fang 1996; Kanbe 1999, Fang 1999) co-localised with trypMase and histamine within the secretory granules (Baram 2001), this may apply even to lung tissue mast cells in ILD in SSC as they harbour MMP-9 transcripts (Edwards 2005). Mast cell MMP-9 production may therefore be determined by relative concentrations of TGFβ, TNFα and SCF (Edwards 2005). The mast cell seems to produce TIMP-1 at low levels constitutively (Baram 2001). Chymase and trypMase increase MMP-9 activity. While trypMase induces neutrophil MMP-9 synthesis and activates MMP-3, which in turn activates MMP-9, chymase activates free and TIMP-1 complexed MMP-9 directly and inactivates TIMP-1 (Frank 2001). As shown in chymase deficient mice, this enzyme may be of special importance to MMP-9 mediated turn-over of fibronectin and collagen in the lung (Tchougounova 2005). There are indications that even histamine may influence MMP-9 levels (Shimizu 2006).

**LYMPHOCYTE ACTIVATION IN SYSTEMIC SCLEROSIS**

**Introduction** In 1986 Mosmann et al first mounted the paradigm of the Th1/Th2 dichotomy, which defines two sets of CD4+ Th cells, producing cytokines promoting cell mediated- and humoral immune response, respectively, and downregulating each others effects (Mosmann 1986). While Th1 cells produce IFNγ, IL-2 and TNFβ and promote the activation of phagocytes and T cytotoxic (c) cells, Th2 cells through the synthesis of IL-4, 5, 6, 10 and 13 stimulate B cell proliferation, maturation and switch to IgE synthesis. The Th1 response clears intracellular- and the Th2 response extracellular infections. Th2 cytokines attract and activate eosinophils and mast cells. Both kinds of Th reaction influence extracellular matrix remodelling. Grossly, Th1 cytokines inhibit fibroblast proliferation, maturation and extracellular matrix production, while Th2 cytokines promote it.

Besides host defence, Th1 and Th2 cells are thought to drive allergic and auto-immune reactions. Th2 may drive allergic diseases, dominated by eosinophilia and IgE synthesis and systemic
diseases with widespread organ engagement and auto-antibody production like SSc, while organ-specific auto-immune diseases like diabetes mellitus may be Th1- driven. However, in most conditions,

there is often a mixed Th1/Th2 reaction, depending on the time course of the disease and the defense reaction mounted (Meyaard 1996).

In recent years new subsets of T cells have been defined (figure 7). Thus the regulatory T cell (Treg) and the Th17 cell may relate to each other in a similar dichotomous way as the Th1 and Th2 cells. The Treg, which expresses the IL-2 receptor z chain constitutively, is thus CD4+CD25+, acts to maintain self-tolerance and to suppress immune responses through the secretion of IL-10 (Tr1 cell) and TGFβ (Th3 cell) (Sakaguchi 2008). The Th17 cells mainly produce IL-17, IL-22 and TNFα and may drive organ-specific auto-immune diseases. Treg- and Th17 cells arise in a mutually exclusive fashion. In the normal state TGFβ will induce Treg cells to maintain self-tolerance, while in a state of infection or inflammation TGFβ and IL-6 combined promote a Th17 response. Antigen specific Th17 cells invade the target tissue first and secrete IL-17, which activates chemokine genes to prepare for Th1- and inflammatory cell invasion (Bettelli 2007, review).

Circulating lymphocytes In SSc, cytokine production by peripheral blood cells is probably skewed towards a Th2 reaction. However, an early report stated a mixed Th1/Th2 reaction with overweight towards a Th2 reaction, that is increased levels of IL-2 and IL-2R in SSc sera, increased IL-2 and IL-4 production by stimulated PBMC and increased supernatant B cell stimulating activity (Famulario 1990). These findings are supported by an increased frequency of raised circulating IL-2, IL-4 and IL-6, with a tendency for elevated IL-4 and IL-6 levels to be associated (Needleman 1992). An increased number of IL-4 producing peripheral CD4+ Th and CD8+ Tc cells and an increased ratio of IL-4/IL-2 CD4+ Th cells in early disease as well as raised soluble CD30 relating to skin score in early dSSc, indicate initial Th2 dominance (Tsuji-Yamada 2001, Giacomelli 1997, Mavilia 1997). Furthermore, elevated circulating levels of IL-10, -13 and -4 (Hasegawa 1997), undetectable circulating IFNγ (Needleman 1992) and low IFNγ production rate by PBMC (Kantor 1992) support the understanding of a probably pathogenetic Th2 reaction in SSc.

Findings, speaking in favour of a mixed Th1/Th2 -or a predominant Th1 reaction, are increased IFNγ and absent IL-4 transcription in PBMC (Gruschwitz 1997). Adding to this, circulating levels and spontaneous production by PBMC of IL-12, a growth factor for Th1 cells, seem elevated in SSc, relating to signs of renal vascular damage (Sato 2000). When examining the intracellular cytokine profile in blood Th cells, half of the populations show Th1 activation (IL-2, IFNγ) and the other half a mixed Th1/Th2 cytokine profile (IL-2, IL-4 and IFNγ) (Valentini 2001). Furthermore, circulating levels of

Figure 7


Host defense (intracellular pathogens) Autoimmunity

Differentiation of CD4+ T cell lineages. Naive peripheral CD4+ T cell precursor cells (THP) can differentiate into three subsets of effector T cells (TH1, TH2 and TH17) and several subsets of regulatory T cells, including induced Treg cells (iTreg), Tr1 cells and Th3 cells and naturally occuring Treg cells (nTreg). The differentiation of these subsets is governed by selective cytokines and transcription factors and each subset accomplishes specialized functions.
IL-17 are elevated and as IL-17 transcripts in peripheral lymphocytes are common in early disease, the Th17 cell may play a role in disease induction (Kurasawa 2000).

**Lymphocytes in interstitial lung disease** In ILD in SSc the alveolar interstitial inflammatory infiltrate is composed of CD3⁺CD45RO⁺ memory T cells (Wells 1995), and associated with the recruitment of neutrophil and eosinophil leukocytes, mast cells and macrophages. Two studies have addressed the question of Th1/Th2 activation in ILD in SSc. In lung tissue a mixed Th1/Th2 reaction dominated, although the number of cells expressing Th1 cytokine mRNA (IL-2, IFNγ) and Th2 cytokine mRNA (IL-4, IL-5) was 20 times greater than in controls, the ratio between Th1 and Th2 cells was normal (Majumdar 1999). In the other study, rapidly progressive ILD in SSc was associated with Th2 cytokine gene expression (IL-4 and/or IL-5 with or without IFNγ) in alveolar CD8⁺ Tc cells, while cells in slowly progressive disease and controls produced IFNγ transcripts, exclusively (Atamas 1999). Lately, IL-17 mRNA has been shown in BALF lymphocytes, however, the role of the Th17 cell in SSc lung disease is uncertain (Kurasawa 2000).
THE AIMS OF THIS THESIS

In this thesis, I have focused on important mechanisms in the development of damage to the vascular system and lung tissue in SSc, organ damage central to morbidity and mortality in this disease. The aspects I have focused on are:

• The synthesis of NO as a measure of endothelial function and its relation to other measures of endothelial cell activation: the adhesion molecules E-selectin, VCAM-1 and ICAM-1.

• The endothelial - dependent and independent vascular function and stiffness of the vascular wall in the brachial and radial arteries in SSc and the relation between these parameters and circulating levels of markers of endothelial cell activation: soluble adhesion molecules and degradation products of NO.

• The relation between bronchoalveolar fluid levels of MMP-9 and its natural inhibitor: TIMP-1 in ILD in SSc and its impact on lung function.

• Possible engagement of the bronchial epithelium and submucosa in SSc and the relation to the presence of ILD and decrease in lung function.

• Possible initiating factors in ILD in SSc: The type of alveolar T cell- and macrophage reaction.
SUBJECTS AND METHODS

Patients (table 8) 33 patients fulfilling the 1982 ARA preliminary criteria for SSc were consecutively included in the studies of this thesis. 27 patients participated in the study described in paper I and of these 24 patients also participated in the investigations described in paper II. While of the 23 women participating in the study described in paper I, 20 also participated in the study described in paper II, all 4 men participated in both the studies. In both studies 2 women and 2 men had dSSc, while the rest suffered from ISSc. Of the 27 patients, 9 with ISSc had ACA, 2 patients with ISSc and 1 with dSSc had Scl-70 antibodies, while 4 patients had high titer RNP antibodies at the time of investigation. Of these, 1 ISSc patient with ACA and 1 with high titer RNP antibodies did not participate in the investigations described in paper II. In the 27 patients and in the subgroup of 24 patients the mean ± SD disease duration from the time of diagnosis to study entry was 12.7± 9.8 years and 13.6 ± 10.5 years, respectively (range 1-38 years for both) and the mean age ± SD at study entry 56.9 ± 14.9 years and 57.9 ± 15.1 years, respectively (range 26-78 years for both). All but 1 patient had Raynaud's phenomenon. Skin score was assessed according to the modified Rodnan model C with 8 unilateral sites and a maximum of 16 points, which resulted in a mean ± SD score of 2.3 ± 1.9 in the group of 27 patients and 2.2 ± 1.7 in the subgroup of 24 patients. 2 patients, both with dSSc had experienced renal crisis with partly restituted renal function (creatinine clearance of 43 and 23 ml/min, respectively).

In the studies on pulmonary engagement in SSc, 16 patients fulfilling the 1982 ARA preliminary criteria for SSc were first consecutively included and described in paper III. 13 patients were females and 3 were men. 12 had ISSc and 4 (2 women and 2 men) dSSc. 9 patients had signs of ILD on HRCT. Anti-Scl-70 antibodies were present in 3 of the patients with ILD and in none of the patients without ILD. ACA were present in 3 patients with ILD and in 4 patients without ILD. The median (IQR) age of the 9 SSc patients with ILD tended to be higher than the age of the 7 SSc patients without ILD (62.0 (13.0) vs. 53.0 (14.0) years). Disease duration median (IQR) tended to be shorter in SSc patients with ILD than in those without ILD (6.0 (9.3) vs. 14.0 (20.8) years). 2 patients with ILD and 2 patients without ILD were ex-smokers. In paper V additional 3 patients were included, while 1 was excluded due to sampling problems. Of the 3 patients included 1 female with dSSc had ILD and Scl-70 antibodies and 2 females with ISSc had normal lungs and ACA. None of them were smokers. In paper IV additional 4 patients were included of whom 2 females with ISSc had normal lungs and ACA. 1 male with ISSc and Scl-70 antibodies, and 1 male with ISSc and ACA had ILD. All 4 patients were smokers.

Controls In all studies healthy age- and sex matched control subjects were randomly selected from within the age cohort in the population registry of the county of Västerbotten, Sweden. In papers I, II and III the number of matched controls equalled the number of SSc patients, while in paper IV and V the number of controls was somewhat smaller than the number of patients, that is 17 and 16 respectively.
Table 8. Patients participating in the studies in this thesis.

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ACA = anticentromere antibodies, ANA = antinuclear antibodies, d = diffuse SSc, l = limited SSc, F = female, ILD = interstitial lung disease, M = male, RNP = antibodies against ribonucleoprotein, Scl-70 = scleroderma 70 antibodies, SSA = antibodies against Sjögren’s syndrome A antigen, SSB = antibodies against Sjögren’s syndrome B antigen.
METHODS
(PAPERS I AND II)

Determination of nitrate levels in plasma and urine Nitrate was measured with a stable isotope ($^{15}$NaNO$_3$) dilution assay utilizing positive ion/chemical ionization gas chromatography/mass spectrometry, after endogenous and labelled nitrate in the samples had been converted to nitrobenzene as described in detail elsewhere (Wennmalm 1993).

Determination of cGMP levels in urine The downstream second messenger element to NO stimulation, cGMP, was measured in urine by a radioimmuno assay using a commercial kit (New England Nuclear, Boston, MA).

Cytokines and adhesion molecules Interleukin-1β, IL-6, IL-1 receptor antagonist (IL-1Ra), IL-10, TNFα, sICAM-1, sVCAM-1, sE-selectin and TGFβ were each measured with specific enzyme-linked immunosorbert assays (ELISA), according to the manufacturer’s protocol. In each assay the appropriate recombinant human cytokine was used to generate the standard curve.

E-selectin expression Skin biopsy specimens from 3 patients and 2 age- and sex matched controls were fixed in paraformaldehyde and then snap-frozen in liquid nitrogen and cut in 5 µm-thick sections. Endogenous peroxidase activity was blocked and appropriate concentrations of anti-E-selectin monoclonal antibody and anti-CD45 applied to the sections. After incubation and washing steps the sections were incubated with peroxidase-conjugated rabbit anti-mouse Ig. Finally the sections were developed with a 3,3' diaminobenzidine tetrahydrochloride and hydrogen peroxide containing solution and counterstained with Gill's hematoxylin.

NO synthase determination Skin biopsy specimens were homogenized for Western blotting. After separation of the homogenates with the NU Page Electrophoresis system, proteins were electroblotted onto polyvinylidene difluoride membranes. The membranes were then incubated with monoclonal antibodies against iNOS and eNOS. Appropriate secondary antibodies conjugated to alkaline phosphatase were used for visualization of the immunoreactive proteins by a chemiluminiscense method, and the reaction detected using hyperfilm.

Measurements of arterial function Endothelium-dependent and endothelium-independent dilation of the right brachial artery was assessed according to the method described by Celermajer et al (Celermajer 1992). Ultrasound scans were performed with a high-resolution ultrasonographic scanner, equipped with a 7.5-Mhz linear-array transducer. The arterial diameter was measured at rest, after reactive hyperemia with increased flow causing endothelium-dependent vasodilation, then again at rest, and finally after sublingual administration of 0.5 mg of nitroglycerin (an endothelium-independent vasodilator). Ultrasound images were recorded on videotape, digitized by a high-resolution frame grabber and stored in a computer. Off-line measurements were performed using dedicated software.

Radial artery stiffness was assessed by pulse applanation tonometry, using a commercially available device (Sphygmo-Cor PX). The system was composed of a signal processing module, a dedicated software system and a pressure tonometer. A transducer (tonometer) held in contact with the radial artery at the wrist received the pulse pattern signal. The system recognized dynamic pulse patterns and calculated the peak systolic pulse pressure derivative (dP/dt$_{max}$), which is a reliable measure of the stiffness of the radial artery.

Statistical analysis Wilcoxon’s signed rank test was used for testing differences between patients and matched controls for continuous data. Differences between 2 independent groups were analyzed with the Mann-Whitney nonparametric test for continuous data and by chi-square test for categorical data. Spearman’s rank correlation test was used for testing correlations between variables. Factor analysis was performed with Quartimax with Kaiser normalization as a rotation method. All P-values refer to 2-sided tests; P values less than 0.05 were considered significant.

(PAPERS III, IV AND V)

Broncho-alveolar lavage

Bronchoscopies Fiberoptic bronchoscopy (Olympus BF type IT200, Tokyo, Japan) with BAL was conducted as follows: In summary, 3 x 60 ml of sterile phosphate buffered saline (PBS), pH 7.3, at 37°C was instilled into the segmental bronchus of the middle lobe of the right lung. The immediately recovered aspirate was collected into a siliconised container placed on ice. Endobronchial mucosal
biopsy specimens were collected either from the anterior aspect of the main carina and the subcarinae of the 3rd and 4th generation airways of the right side or from the posterior aspect of the main carina and the corresponding subcarinae on the left side.

**Processing of lavage fluid and cells** The aspirated BAL fluid was passed through a nylon filter (pore diameter 100 micro m, Syntab Product AB, Malmö, Sweden) and centrifuged at 400 G for 15 minutes to remove mucus and cellular components. Supernatants were separated from cell pellets and the cell free fluid divided into aliquots and stored at – 80°C prior to analysis. Cell pellets derived from BAL were re-suspended in PBS to give a final concentration of 10^6 cells/ml and total and differential leucocyte counts performed. The total number of cells in the lavage fluid was counted in a Bürker chamber. Cytocentrifuged specimens with 5 x 10^4 non-epithelial cells per slide were prepared using a cytospin 3 (Shandon Southern Instruments Inc., Sewikly, PA, USA) 1,000 rpm (96 G) for 5 minutes. Cell differential counts were conducted on slides stained according to May-Grünwald Giemsa and 400 cells per slide were counted. Mast cells were counted in at least 10 visual fields at x 160 magnification on slides stained with acid toluidine blue and counter stained with Mayer’s acid haematoxylin.

**Fixation and immunohistochemical staining of lung biopsy samples** In this study, we investigated whether inflammation-sensitive transcription factors were activated in the bronchial epithelium. Immunohistochemical staining was used to quantify the expression of the transcription factors: nuclear factor kappa B (NFκB) (p65), and activation protein (AP)-1 (c-jun and c-fos), as well as their upstream mitogen activated protein kinases (MAPKs): p38, JNK and ERK. Moreover, the neutrophil attracting chemokines: IL-8, ENA -78 and Gro-α were investigated by immunostaining of the epithelium.

In the bronchial submucosa, endothelial expression of adhesion molecules: platelet (P)-selectin and ICAM-1as a sign of inflammation and endothelial activation was investigated as well as inflammatory cell infiltration (neutrophils, mast cells, CD3+ T lymphocytes, CD4+ Th- and CD8+ Tc cells).

Briefly, endobronchial mucosal biopsy samples were fixed overnight in a solution containing chilled acetone and protease inhibitors before processed into glycol methacrylate (GMA) resins. The fixed biopsies were cut in two micron thin slices and placed on glass slides. Endogenous peroxidases were inhibited and non-specific antibody binding was blocked before the primary antibodies were applied and incubated over night. Finally, a biotinylated secondary antibody was added before the sections were developed and counterstained.

**Evaluation of immunohistochemical staining** The immunoreactivity of the staining was evaluated using a light microscope and computer-assisted image analysis equipment. The image analysis equipment included a colour video camera connected to an imaging work station with dedicated software.

**Cells** The quantification of positively stained immune cells was carried out using a light microscope. Cells were counted separately in the epithelium and the submucosa, excluding mucosal glands, blood vessels and smooth muscle. The length of the epithelium and the area of the submucosa were quantified using the computer-assisted image analyser. In the epithelium the counts were expressed as cells/mm and in the submucosa as cells/mm².

**Cytokines** The cytokine expression in intact areas of the bronchial epithelium was quantified using the computer assisted image analyser. The cytokine expression was given as percentage of positively immunostained area out of the total epithelial area.

**Adhesion molecules** Quantification of endothelial adhesion molecule expression was done by expressing the number of blood vessels stained with a specific anti-adhesion molecule mAb, as a percentage of the total vessel complement, revealed by the staining with the pan-endothelial mAb EN4.

**Transcription factors** MAPKs and transcription factor immunoreactivity was quantified using the computer assisted image analyser. The immunoreactivity was determined as positive cytoplasmic plus nuclear (total) staining, given as a percentage of the epithelial area selected with the image system. Nuclear translocation was determined as the number of positively stained nuclei/mm² of bronchial epithelium detected by light microscopy.

**MMP-9 and TIMP-1** Cleavage of pro-MMP-9 near or at residue 87 results in the active enzyme with a mass of approximately 82 kDa. Levels of total-MMP-9 (active + pro-MMP-9) were measured by means of an ELISA assay (R&D Systems, Oxon, UK). The detection limit for total MMP-9 was 0.156 ng/ml. Levels of pro-MMP-9 were analysed using an ELISA method (Amersham Pharmacia, Buckinghamshire, UK). The values recorded included free and TIMP-complexed forms. For the pro-MMP-9 ELISA the detection limit was 0.06 ng/ml. Active MMP-9 values were calculated as the difference between concentrations of total MMP-9 and pro-MMP-9.
Levels of TIMP-1 were measured using an ELISA method (Amersham Pharmacia, Buckinghamshire, UK). The values recorded included free and MMP-bound forms. For the TIMP-1 ELISA the detection limit was 1.25 ng/ml.

**Myeloperoxidase (MPO)** MPO is released from activated neutrophils and is therefore considered a marker of neutrophil activation. MPO was measured by radio-immuno assay (RIA) (Pharmacia AB, Uppsala, Sweden). The detection limit was 8 µg/L.

**Methyl-histamine** Histamine is synthesised by mast cells and basophils and stored in secretory granules. Released histamine disappears within minutes due to methylation via histamine-N-methyltransferase. Methyl-histamine was measured by RIA (Pharmacia AB, Uppsala, Sweden). The detection limit was 0.1µg/L.

**Eosinophilic Cationic Protein (ECP)** ECP is a strikingly basic protein localised in the eosinophil granule matrix and a member of the ribonuclease gene superfamily. ECP was measured by RIA (Pharmacia AB, Uppsala, Sweden). The detection limit was 1 µg/L.

**Isolation of PBMC and BALF T lymphocytes and macrophages and RNA extraction** Peripheral blood- and alveolar T cells as well as peripheral blood monocytes and alveolar macrophages were separated from blood and lavage samples using positive selection with immunomagnetic beads coated with anti-CD3 mAb and anti-CD14 mAb, respectively. Total RNA was isolated from lysates of these cell populations.

**RT-PCR** Single strand cDNA copies were made from 1 µg of total RNA. Subsequent PCR amplification for IL-1β, IL-2, -3, -4, -5, -6, -7, -8, -10, IFNγ, TNFα, TNFβ, GM-CSF, CD45RO, and beta-actin was performed and the PCR products analyzed in agarose gel electrophoresis, stained with ethidium bromide and visualized by UV illumination.

**Lung function assessments** Lung volumes, dynamic spirometry and DLco were measured according to standard procedures (Master Spirometer and Master Pro Transfer; Jaeger, Würzburg, Germany).

**High resolution computed tomography of the lungs** HRCT of the lungs was performed using a Philips Tomoscan LX Single slice scanner (MA, USA). Scans were performed at full inspiration in the supine position with 120 kV, 175 mAmp including contiguous scans throughout the lungs with 10 mm thickness followed by scans with 1.5 mm thickness with a slice spacing of 30 mm. Ground glass opacities, reticulate pattern fibrosis, non-septal and/or subpleural lines and honeycombing were considered signs of ILD (Devenyi 1995).

**Statistical analysis** Results were reported as median and in terquartile range (IQR). Comparisons between the 3 groups (SSc patients with ILD, SSc patients without ILD and controls) were analysed with a non-parametric ANOVA (Kruskal-Wallis test). A significant difference was considered at the 5% level. If a significant change was found in a variable using the Kruskal-Wallis test, post-hoc analyses were performed using Mann-Whitney U-test. The Mann-Whitney non-parametric test was also used to compare lung function variables between SSc patients with and without ILD. Correlation analyses were performed using the non-parametric Spearman’s rank correlation test. P values less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

**PAPER I**

In this paper we showed that circulating levels of nitrate is elevated in SSc patients and that the site of the increased production probably is the endothelial cells. The finding of increased urinary excretion of cGMP, the second downstream messenger of NOS activation corroborated the notion of increased NO production. Cyclic GMP is produced in the VSMC by guanylate cyclase upon its activation by NO and acts on cGMP protein kinase to accomplish VSMC relaxation via K+ channel activation and increase in intracellular Ca2+. Furthermore, we found elevated levels of soluble endothelial adhesion molecules: E-selectin, VCAM-1 and ICAM-1 also pointing at endothelial activation (figure 1). Of these adhesion molecules, E-selectin is probably only expressed and shed from endothelial cells, while VCAM-1 may also be expressed by epithelial cells, monocytes/macrophages and dendritic cells and ICAM-1 may be shed by an even greater number of differing cell types including fibroblasts.
Figure 1. Box plots showing the levels of plasma nitrate (a), urine nitrate (b), urine cGMP (c), soluble intercellular adhesion molecule 1 (sICAM-1) (d), soluble vascular cell adhesion molecule 1 (sVCAM-1) (e), soluble E-selectin (f), interleukin 6 (IL-6) (g), IL-10 (h), transforming growth factor β1 (TGFβ1) (i), and IL-1 receptor antagonist (IL-1Ra) (j) in patients with systemic sclerosis (n = 27) and age- and sex-matched controls. Boxes show the 25th and 75th percentiles; horizontal lines in boxes show the medians; bars above and below boxes show the 10th and 90th percentiles; open circles show outlying values. *P* values were determined by Wilcoxon's signed rank test.
We found a significant correlation between levels of plasma nitrate and soluble adhesion molecules sE-selectin and sVCAM-1, indicating that the activated endothelium may produce increased amounts of NO as well as adhesion molecules (table 2). To further explore the possibility of a common origin of adhesion molecules and NO, we performed factor analysis. The exploratory factor analysis, when all variables were included, showed clustering of plasma nitrate, sE-selectin, sVCAM-1 and sICAM-1 on one factor, which may be considered to represent the endothelium, and thus the result was consistent with the conclusion that activated endothelial cells are a likely source of the increased plasma nitrate (table 4).

**Table 2.** Spearman’s rank correlation coefficients for correlations between levels of p-NO, u-NO, urine cGMP, soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), and IL-6 in 27 patients with systemic sclerosis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>u-NO</th>
<th>cGMP</th>
<th>sICAM-1</th>
<th>sVCAM-1</th>
<th>sE-selectin</th>
<th>IL-6</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-NO</td>
<td>0.648†</td>
<td>0.047</td>
<td>0.384</td>
<td>0.465‡</td>
<td>0.472‡</td>
<td>0.207</td>
</tr>
<tr>
<td>u-NO</td>
<td>0.132</td>
<td></td>
<td>0.092</td>
<td>0.246</td>
<td>0.191</td>
<td>0.179</td>
</tr>
<tr>
<td>cGMP</td>
<td></td>
<td></td>
<td>0.130</td>
<td>0.056</td>
<td>−0.089</td>
<td>0.068</td>
</tr>
<tr>
<td>sICAM-1</td>
<td></td>
<td></td>
<td></td>
<td>0.554†</td>
<td>0.636†</td>
<td>0.360</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.623†</td>
<td>0.652§</td>
</tr>
<tr>
<td>sE-selectin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.239</td>
</tr>
</tbody>
</table>

† Plasma nitrate (p-NO) and urine nitrate (u-NO) were used as indicators of nitric oxide. sICAM-1 = soluble intercellular adhesion molecule 1; sVCAM-1 = soluble vascular cell adhesion molecule 1; IL-6 = interleukin-6.
‡ P < 0.01.
§ P < 0.001.

**Table 4.** Factor analysis with principal component analysis for p-NO, u-NO, urine cGMP, soluble adhesion molecules (sICAM-1, sVCAM-1, and sE-selectin), IL-6, and creatinine clearance in 27 patients with systemic sclerosis.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Factor 1</th>
<th>Factor 2</th>
<th>Factor 3</th>
<th>Factor 4</th>
</tr>
</thead>
<tbody>
<tr>
<td>p-NO</td>
<td>0.441</td>
<td>0.803</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>u-NO</td>
<td>−</td>
<td>0.931</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>cGMP</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.933</td>
</tr>
<tr>
<td>sICAM-1</td>
<td>0.857</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>0.647</td>
<td>−</td>
<td>0.614</td>
<td>−</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>0.902</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>IL-6</td>
<td>−</td>
<td>−</td>
<td>0.939</td>
<td>−</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>−0.662</td>
</tr>
</tbody>
</table>

* See Table 2 for definitions.

To further verify the presence and plausibility of the above assumption of the activated endothelium as a source of NO, we examined skin biopsies for the presence of E-selectin staining. Moreover, we examined the skin biopsies for the presence of iNOS and eNOS protein by means of Western blotting. In the subepidermal portion of the skin we found capillaries expressing E-selectin on their luminal side. Moreover, these capillaries were surrounded by leukocytes, which may have migrated from the vessel by means of interaction with the adhesion molecules investigated (figure 3). In the Western blotting analysis we found iNOS and eNOS expressed in SSc skin (figure 2).
Figure 3. Immunoperoxidase staining for E-selectin (A and B) and CD45 antigen (D) and control staining (C). A, Skin specimen showing 4 dermal capillaries (arrows), stained with monoclonal antibody (mAb) against E-selectin. E = epidermis. B, Capillary in the subepidermal portion of the dermis, showing E-selectin-positive staining of the endothelial surface facing the lumen of the vessels (arrows) and in close contact with the endothelial cells. MC = mononuclear cells. C, Staining with isotype matched inappropriate mAb (negative control). D, Cross section of skin from the same patient as in B, showing a group of blood vessels (V), surrounded by leukocytes, stained with mAb CD45 (arrows).

Table 2. Western blotting of skin biopsy samples from 3 SSc patients (P1, P2, P3) and 1 healthy control subject (Ctrl). Arrows indicate bands of endothelial nitric oxide synthase (eNOS) and inducible nitric oxide synthase (iNOS). The 1 patient showing marked staining for both eNOS and iNOS was a 49-year old woman with dSSc since 3 years. The biopsy specimen was from her upper arm. Std enz = standard enzyme (a strongly positive reference).

<table>
<thead>
<tr>
<th>Panel</th>
<th>Patient</th>
<th>Ctrl</th>
<th>Std enz</th>
</tr>
</thead>
<tbody>
<tr>
<td>eNOS</td>
<td>P1</td>
<td>P2</td>
<td>P3</td>
</tr>
<tr>
<td>116 kDa</td>
<td>97 kDa</td>
<td></td>
<td></td>
</tr>
<tr>
<td>iNOS</td>
<td>P1</td>
<td>P2</td>
<td>P3</td>
</tr>
<tr>
<td>116 kDa</td>
<td>97 kDa</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
It is now generally agreed upon, that iNOS may be demonstrated in several cell types such as mononuclear cells, fibroblasts and endothelial cells in SSC. Both mononuclear cells (Yamamoto 1998) and fibroblasts (Takagi 2003) from SSC patients have been shown to express iNOS and to produce increased amounts of NO, probably due to the induction of iNOS by pro-inflammatory cytokines. However, probably because of the absence of a functioning method to culture human endothelial cells, it has not been possible to measure NO production in SSC endothelial cells, directly. Therefore, in SSC, most studies have been restricted to the demonstration of the expression of eNOS, iNOS and nitrotyrosine in endothelial cells in skin biopsies, by immunohistochemical-, RT-PCR- and Western blotting methods (Yamamoto 1998, Cotton 1999, Dooley 2006).

Since the acceptance and publication of our works, several articles on the issue of NO production in SSC have been published. Among these, some trying to enlighten the role of the endothelial cell in NO production in SSC. Pertainig to this issue is the study by Cotton et al 1999, which is an important immunohistochemical study on the endothelial expression of eNOS and iNOS and the accumulation of nitrotyrosine in histologically graded SSC skin. The study pointed out the loss of eNOS and the increase of iNOS with increasing inflammatory changes. Moreover, with increasing fibrosis, iNOS expression decreased and nitrotyrosine deposition increased reciprocally –probably as a result of protein tyrosine residue nitration by peroxynitrite. These changes were present in superficial and deep capillaries and venules. The arterioles -in contrast to the findings in arterioles of normal skin- lacked eNOS, however, neither iNOS could be detected. The findings support our notion of endothelial activation in SSC.

Recently, a more complex and seemingly inconsistent work on the endothelial production of NO in SSC has been published (Dooley 2006). In this study nitrotyrosine was found depositcd especially around microvessels near fibrous deposits. This finding is in agreement with the findings by Cotton et al and supports our notion of increased endothelial NO production. In the same study, SSC serum was found to inhibit NOS activity, that is NO and citrulline production, to lower cGMP levels and leave eNOS and iNOS expression unaffected in cells from a cultured human dermal endothelial cell line. This was a surprising result as the induction of iNOS in endothelial cells is stimulated by the pro-inflammatory cytokines IL-1β, TNFα and IL-6, of which IL-1β and TNFα have been found to be produced spontaneously by PBMC in SSC (Kantor 1992), while IL-6 levels have been found elevated in SSC serum (Needleman 1992). The discrepancy may be explained by the fact, that endothelial cells, in vivo, probably are stimulated by much higher cytokine levels produced by close infiltrating monocytes, than the levels found in SSC serum. Furthermore, the reciprocal activation and inhibition of eNOS and iNOS is complicated and may partly explain the above findings (Colasanti 1997, 1999).

Several articles reporting the results of measurement of circulating levels of NO metabolites have appeared since our data were published (Sud 2000, Allanore 2001, Takagi 2003, Dooley 2006). Most authors have in agreement with our measurements found raised levels of NO metabolites in SSC (Sud 200, Takagi 2003, Dooley 2006), while Allanore et al showed lowered circulating levels. However, there are no further data correlating circulating NO metabolite levels and markers of endothelial activation.

Some authors have tried to correlate NO levels to the presence of certain disease manifestation in SSC, thus Takagi et al reported the highest NO metabolite levels in patients with early dSSC with signs of ILD. These associations were, however, not confirmed by Sud et al, who found elevated nitrite and citrulline levels not influenced by the presence of ILD or correlated to skin score. Dooley et al found the highest levels of circulating nitrate and nitrite in patients with limited skin engagement and extremely high levels of nitrotyrosine on plasma proteins in early diffuse disease. In our study nitrate levels did not correlate to skin score or the presence of ILD. These findings may have been influenced by our relatively small number of patients.

Studies on exhaled NO in SSC also deal with the subject of endothelial NO synthesis in SSC. The sources of exhaled NO in SSC are several. In SSC patients without ILD, demonstrated raises in exhaled NO may be caused by increased systemic production. In SSC with ILD increased exhaled NO may be generated by macrophages, epithelial cells, neutrophils and activated endothelial cells in the lung tissue (Saleh 1997). In SSC with IPHT, NO production might be expected to be increased in the endothelial cells of the pulmonary vascular tree. Probably because of the relatively small number of SSC patients affected with IPHT, studies are few and comprise only small numbers of patients. In a study on 47 SSC patients, the 5 patients with IPHT had lower levels of expired NO than patients with normal lungs and healthy controls although these differences were not significant (Rolla 2000). These results are in agreement with the findings in early studies by Kharitonov et al and Rolla et al, who showed reduced levels of expired NO in 6 and 2 SSC patients with PHT, respectively (Kharitonov 1997, Rolla 1998). Exhaled NO in IPHT in SSC may be low for several reasons. At first, it is reasonable to assume that the diffusion of NO in patients with a low DLCO also is lowered due to thickening of the
vascular walls (Girgis 2002). Secondly, exhaled NO is low in patients with reduced cardiac output, as it might be in patients with PHT (Sumino 1998). That is, theoretically, pulmonary endothelial NO production might actually be increased in IPHT in SSc, but due to vascular wall thickening, the increased synthesis does not result in increased exhaled NO. Thirdly, pulmonary endothelial NO production may not be uniform as some vascular beds may be so deranged that endothelial cells do not function normally (Saleh 1997).

**PAPER II**

Since our article on the vascular function of the brachial and radial arteries was published no further examinations of the function of these two arteries have been published to my knowledge. However, these vessels have been the object of an arteriographic study showing that in SSc patients with digital ulceration or gangrene, occlusion of the digital arteries is common, occlusion of the palmar arch and ulnar artery is occasionally present, while no patient showed occlusion of the radial artery (Hasegawa 2006). The result of the study was in close agreement with earlier findings (Stucker 2000).

In our study, we found increased stiffness—or decreased compliance—of the radial arterial wall in SSc patients. Furthermore, our measurements showed normal endothelium-dependent as well as normal endothelium-independent dilation of the brachial artery (figure 1).

The stiffness of the radial artery correlated significantly to levels of plasma nitrate and sVCAM-1, indicating that endothelial activation (or inflammation) increases the radial arterial stiffness. Despite the findings of normal dilation of the brachial artery in response to increased flow (flow mediated dilation (FMD%), which is endothelium-dependent and to nitroglycerin intake, which is endothelium-independent (NTG%), the results of the correlation analysis spoke in favour of brachial artery involvement in SSc. Statistical analysis, namely, showed significant inverse correlations between FMD% and sE-selectin and between NTG% and plasma nitrate, sE-selectin and sVCAM-1.

![Figure 1](image)

**Figure 1.** Box plots showing A, maximum rise in pulse pressure per second (dP/dt\text{max}) in the radial artery, B, flow-mediated dilation (FMD%; % change from baseline) of the brachial artery, and C, nitroglycerin-induced dilation (NTG%; % change from baseline) of the brachial artery in patients with systemic sclerosis (shaded boxes) and in age- and sex-matched healthy control subjects (open boxes) (n = 24 subjects per group for A and B; n = 20 subjects per group for C). Boxes show the 25th and 75th percentiles; horizontal lines within the boxes show the median; bars above and below the boxes show the 10th and 90th percentiles; open circles show outlying values. $P$ values were determined by Wilcoxon's signed rank test.
That is, the results of the correlation analysis indicated that endothelial activation (or inflammation) might have a restricting effect, deciding the flow mediated- and nitroglycerin induced level of dilation of the brachial artery (table 2, figure 3). This may raise the suspicion that the high-resolution ultrasound method we used to measure changes in brachial artery diameter was too insensitive to show small variations in diameter. Moreover, a greater number of patients included might have equipped the study with more power and changed the result. This interpretation is further supported by the finding by Moyssakis et al 2005 of decreased distensibility of the ascending aorta in SSc, showing that vascular changes in SSc may not be confined to small forearm or digital vessels.

Table 2. Correlations between plasma nitrate levels, soluble endothelial adhesion molecule levels, radial artery wall stiffness and brachial artery dilation and diameter in 24 SSc patients and 24 age- and sex-matched healthy controls*

<table>
<thead>
<tr>
<th></th>
<th>sE-selectin</th>
<th>sVCAM-1</th>
<th>Radial artery wall stiffness, by dP/dt_{max}</th>
<th>Brachial artery dilation</th>
<th>Brachial artery diameter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>By FMD%</td>
<td>By NTG%†</td>
</tr>
<tr>
<td>SSc patients</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma nitrate</td>
<td>0.472‡</td>
<td>0.465‡</td>
<td>0.506‡</td>
<td>−0.202</td>
<td>−0.697§</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>0.623‡</td>
<td></td>
<td>0.288</td>
<td>−0.400‡</td>
<td>−0.505‡</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>0.487‡</td>
<td></td>
<td></td>
<td>−0.342</td>
<td>−0.555‡</td>
</tr>
<tr>
<td>Radial artery wall stiffness, by dP/dt_{max}</td>
<td></td>
<td></td>
<td></td>
<td>−0.295</td>
<td>−0.474‡</td>
</tr>
<tr>
<td>Brachial artery dilation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By FMD%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By NTG%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Healthy controls</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma nitrate</td>
<td>0.176</td>
<td>0.061</td>
<td>−0.075</td>
<td>−0.280</td>
<td>−0.016</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>0.035</td>
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<td>0.115</td>
<td>0.368</td>
<td>0.116</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>−0.264</td>
<td></td>
<td>−0.028</td>
<td>0.057</td>
<td></td>
</tr>
<tr>
<td>Radial artery wall stiffness, by dP/dt_{max}</td>
<td></td>
<td></td>
<td></td>
<td>−0.040</td>
<td>−0.217</td>
</tr>
<tr>
<td>Brachial artery dilation</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By FMD%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>By NTG%</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>−0.583§</td>
</tr>
</tbody>
</table>

* Correlation coefficients were determined by Spearman’s rank correlation. Radial artery wall stiffness was measured as the maximum increase in systolic pulse pressure over time (dP/dt_{max}). Endothelium-dependent (i.e. flow-mediated) dilation (FMD%) and endothelium-independent (i.e. nitroglycerin-induced) dilation (NTG%) of the brachial artery were measured as the percentage of change from baseline.

sE-selectin = soluble E-selectin; sVCAM-1 = soluble vascular adhesion molecule 1; SSc = systemic sclerosis.

† Measured in 20 SSc patients.
‡ P < 0.05.
§ P < 0.01.

Table 3. Factor analysis for brachial artery dilation by NTG%, plasma nitrate levels, soluble endothelial adhesion molecule levels, radial artery wall stiffness, brachial artery diameter, age, and creatinine clearance in 20 patients with SSc*

<table>
<thead>
<tr>
<th></th>
<th>1</th>
<th>2</th>
<th>3</th>
<th>4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brachial artery dilation, by NTG%</td>
<td>−0.421</td>
<td>−</td>
<td>−0.555</td>
<td>−0.510</td>
</tr>
<tr>
<td>Plasma nitrate</td>
<td>0.552</td>
<td>−</td>
<td>0.531</td>
<td>−</td>
</tr>
<tr>
<td>sE-selectin</td>
<td>0.828</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>sVCAM-1</td>
<td>0.874</td>
<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>Radial artery wall stiffness, by dP/dt_{max}</td>
<td>−</td>
<td>−</td>
<td>0.962</td>
<td>−</td>
</tr>
<tr>
<td>Brachial artery diameter</td>
<td>−</td>
<td>−</td>
<td>−</td>
<td>0.906</td>
</tr>
<tr>
<td>Age</td>
<td>−</td>
<td>0.522</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>Creatinine clearance</td>
<td>−</td>
<td>0.705</td>
<td>−</td>
<td>−</td>
</tr>
</tbody>
</table>

* The factor extraction method used was principal components. The extraction rule used was greater than one. The transformation method used was orthotran/varimax. See table 2 for definitions.
Figure 3. Scattergrams showing the correlations (Spearman’s rank correlation coefficients) between nitroglycerin-induced dilation of the brachial artery and levels of A, plasma nitrate (patients $r_s = -0.697; P = 0.0018$; controls $r_s = 0.003, P = 0.9911$; all $r_s = -0.269, P = 0.0889$), B, soluble E-selectin (sE-selectin) (patients $r_s = -0.505, P = 0.0276$; controls $r_s = 0.111, P = 0.6275$; all $r_s = -0.193, P = 0.2213$), and C, soluble vascular cell adhesion molecule 1 (sVCAM-1) (patients $r_s = -0.555, P = 0.0156$; controls $r_s = 0.072, P = 0.7530$; all $r_s = -0.185, P = 0.2421$) in 20 systemic sclerosis patients (●) and 20 age- and sex-matched healthy controls (○).

Furthermore, we performed exploratory factor analysis to seek latent factors explaining the inverse correlation between plasma nitrate and the NTG% as nitrate tolerance has been described in murine VSMC exposed to chronic stimulation by NO produced by overexpressed eNOS (Yamashita 2000). We
identified 4 factors including one showing large loadings on plasma nitrate, sE-selectin, VCAM-1 and negative loading on NTG%, which all represent measures of endothelial activation. The 2\textsuperscript{nd} factor showed large loadings on age and creatinine clearance, and may represent renal function. The 3\textsuperscript{rd} factor showed large loadings on plasma nitrate, radial arterial stiffness by dP/dt\textsubscript{max} and negative loading on NTG%. The 3\textsuperscript{rd} factor may represent nitrate tolerance (table 3).

Lately, several articles on the function of digital vessels and vessels from the skin of the hand and forearm have been published. These articles fall into three categories: examinations of endothelium-dependent- and independent vascular function, examinations of the response to heating and cooling and examinations of the reaction to vaso-constrictory agents especially with regard to their influence on vascular protein tyrosine kinase (PTK) activity.

Currently, newer more sensitive techniques for measuring changes in blood flow have emerged as for example laser Doppler imaging, which is a non-contact method measuring blood flow in a skin area and dual-wavelength laser Doppler imaging, allowing measurement of the flow in both smaller (capillaries) and larger (thermoregulatory) vessels. Previously, only single-point measurement by laser Doppler flowmetry, where the probe contact might in itself alter blood flow and which only measured the flow in large thermoregulatory vessels was available.

Small numbers of patients and insensitive techniques have often hampered early studies on endothelium-dependent- and independent dilation, giving conflicting results. In recent investigations there has been a trend toward finding impairment of both endothelium-dependent- and independent dilation in finger and forearm skin. Thus, using the new laser Doppler imaging technique in combination with iontophoresis of acetylcholine and nitroprusside, endothelium-dependent as well as independent dilation was found decreased in SSc digital skin by Anderson et al, who previously could not show any difference between SSc patients and controls using laser Doppler flowmetry (Anderson 2004, Anderson 1999, Anderson 2003). These findings further strengthen the concept of disturbed vascular function in finger skin as previously shown by La Civita et al (La Civita 1998). Moreover, in SSc forearm skin a delay in – as well as- a decreased endothelium-dependent dilation has been demonstrated (Marasini 2001).

Recently, it has been appreciated that NO release is partly responsible for the vasodilation achieved by local heating. Thus the vasodilatory response is thought to be mediated by at least two independent mechanisms: an initial peak in cutaneous blood flow during the first 10 minutes is mediated by a calcitonin gene-related peptide (CGRP) and substance P-dependent axonal reflex, followed by a nadir and a secondary NO mediated plateau. In SSc finger pad the initial thermal flow peak is often missing or comes late and has lower amplitude than in controls. Moreover, the NO mediated thermal plateau flow at maximal heating is decreased and delayed, the delay correlating to the delay in peak flow after occlusion (endothelium-dependent dilation) (Boignard 2005). These changes seem to be unrelated to skin fibrosis but might be related to finger blood pressure and the presence of digital pitting scars (Salvat-Melis 2006). In SSc forearm skin (Salvat-Melis 2006) as well as in the capillaries and thermoregulatory vessels of the skin of the dorsum of the hand thermal hyperaemia seems normal (Murray 2006).

Other mechanisms than changes in NO metabolism have been proposed to be involved in vascular hyperreactivity in SSc. Furspan et al have shown that vascular PTK may mediate increased vascular contraction in SSc (Furspan 2004). Several vasoconstrictory agents such as noradrenaline, adrenaline, serotonin and angiotensin II are G-protein coupled and activate PTK pathways. In SSc arterioles dissected from forearm skin, the increased contraction in response to cooling may be reversed by adding antagonists to PTK to the perfusate and increased by adding an agonist. Moreover, SSc vascular wall phosphorylated tyrosine increases more than in controls in response to cooling (Furspan 2005).

The results of our study pointed at an independent inhibiting effect of the chronically elevated NO synthesis in SSc on the ability of the brachial artery to dilate in response to nitroglycerin medication. In recent years the effects of chronically increased NO production due to the presence of iNOS in the endothelial cell has been focused by a great number of authors and their observations have cast light over the biochemical mechanisms causing this phenomenon of endothelial dysfunction or nitrate tolerance. Thus vascular dilation in normal vessels is regulated by the shear stress dependent low-output NO production by eNOS, but in situations of vascular damage leading to inflammation, synthesis of iNOS via activation of NFkB may cause an appreciable rise in NO formation. High-output NO production by iNOS may result in depletion of the substrate arginine and the NOS co-factors calmodulin (Vasquez-Vivar 1998) and BH4 (Landmesser 2003), leading to uncoupling of electron transfer from NO production to the synthesis of superoxide (O_2^-). The resulting O_2^- instantaneously reacts with NO to form peroxynitrite (ONOO^-), a highly reactive free radical able to nitrate protein tyrosine residues and oxidize sulphur bonds.
In the search for the mechanism behind NO tolerance it has been shown that peroxynitrite is able to diminish the vasodilatory action of NO through the modulation of several enzymes. Thus peroxynitrite nitrates soluble guanylate cyclase (Weber 2001), rendering the enzyme incapable to respond to increases in NO levels with the formation of cyclic GMP. Peroxynitrite may even stimulate NFκB nuclear translocation (Cooke 2002), leading to iNOS synthesis and further increase in the unfavourable peroxynitrite production via a positive feed-back mechanism. Moreover, peroxynitrite may inactivate superoxide dismutases (SODs), which catalyze the dismutation of superoxide to oxygen and hydrogen peroxide (Demicheli 2007), further increasing superoxide concentration and the possibility of peroxynitrite formation. Furthermore, peroxynitrite may nitrate prostacyclin synthase to inactivate this other pathway of vasodilation (Hink 2003). Peroxynitrite may also interfere with the breakdown of asymmetric dimethylarginine (ADMA), a by-product of protein degradation during oxidative stress and an inhibitor of all NOS (Leiper 2002). There are several other ways in which peroxynitrite may interfere with NO induced vasodilation. Thus several observations point at peroxynitrite as one link to nitrate tolerance.

**PAPER III**

In this paper we demonstrated significant increases in BALF total MMP-9 and pro-MMP-9 as well as calculated active MMP-9 levels in SSc patients with signs of ILD on HRCT of the lungs. We even showed elevated levels of TIMP-1 in SSc patients both with- and without ILD. Moreover, we found levels of MPO elevated and a tendency to elevated levels of ECP in SSc patients with ILD, strengthening the concept of neutrophil- and eosinophil activation in ILD in SSc (table 3).

What is more, total-, pro- and calculated active MMP-9 as well as the pro-MMP-9/TIMP-1 ratio and the active MMP-9/TIMP-1 ratio all correlated significantly and negatively to TLC in SSc patients, indicating a causal association to the degradation of lung tissue (table 1, figure 1).

**Table 1**

Spearman’s rank correlation test between total lung capacity and MMP-9 derivatives in BALF from 16 patients with SSc.

<table>
<thead>
<tr>
<th></th>
<th>r_s</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MMP-9</td>
<td>-0.68</td>
<td>0.01</td>
</tr>
<tr>
<td>Pro-MMP-9</td>
<td>-0.58</td>
<td>0.02</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>-0.67</td>
<td>0.009</td>
</tr>
<tr>
<td>Pro MMP-9/TIMP-1</td>
<td>-0.56</td>
<td>0.03</td>
</tr>
<tr>
<td>Active MMP-9/TIMP-1</td>
<td>-0.62</td>
<td>0.016</td>
</tr>
</tbody>
</table>

**Figure 1.** Correlations between active MMP-9 and TLC in percent of predicted in 16 systemic sclerosis patients. Solid circles represent patients with interstitial lung disease.
Neutrophil cell count correlated to levels of total- and calculated active MMP-9 levels as well as to ratios between pro-MMP-9/TIMP-1 and calculated active MMP-9/TIMP-1. These findings indicate that the neutrophil is an important source of MMP-9 in ILD in SSc (table 2).

**Table 2**
Spearman’s rank correlation test between neutrophil count and MMP-9 derivatives in BALF from 16 patients with SSc.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SSc without ILD</th>
<th>P vs. controls</th>
<th>SSc with ILD</th>
<th>P vs. controls</th>
<th>P comparison between SSc groups</th>
<th>P Kruskal–Wallis test</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 16</td>
<td>N = 7</td>
<td></td>
<td>N = 9</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>ECP µg/L</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td>Median (IQR)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>MPO µg/L</td>
<td>0.9 (0.5)</td>
<td>1.0 (0.06)</td>
<td>0.8</td>
<td>1.8 (1.4)</td>
<td>0.03</td>
<td>0.03</td>
<td>0.05</td>
</tr>
<tr>
<td>Total MMP-9 ng/ml</td>
<td>3.5 (4.1)</td>
<td>1.6 (3.7)</td>
<td>0.3</td>
<td>6.6 (14.7)</td>
<td>0.004</td>
<td>0.009</td>
<td>0.009</td>
</tr>
<tr>
<td>Pro-MMP-9 ng/ml</td>
<td>0.2 (0.4)</td>
<td>0.08 (0.05)</td>
<td>0.2</td>
<td>0.4 (0.1)</td>
<td>0.03</td>
<td>0.0008</td>
<td>0.009</td>
</tr>
<tr>
<td>Active MMP-9 ng/mL</td>
<td>1.01</td>
<td>0.620</td>
<td>0.0093</td>
<td>2.713</td>
<td>0.057</td>
<td>0.0012</td>
<td>0.002</td>
</tr>
<tr>
<td>TIMP-1 ng/ml</td>
<td>(1.063)</td>
<td>(0.595)</td>
<td>(3.746)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pro-MMP-9/TIMP-1</td>
<td>0.009</td>
<td>0.002</td>
<td>0.07</td>
<td>0.009</td>
<td>0.3</td>
<td>0.005</td>
<td>0.02</td>
</tr>
<tr>
<td>Active MMP-9/TIMP-1</td>
<td>0.055</td>
<td>0.009</td>
<td>0.0013</td>
<td>0.064</td>
<td>0.808</td>
<td>0.0070</td>
<td>0.004</td>
</tr>
</tbody>
</table>

SSc, systemic sclerosis; ILD, interstitial lung disease; IQR, interquartile range; ECP, eosinophilic cationic protein; MPO, myeloperoxidase; MMP-9, matrix metalloproteinase 9; TIMP, tissue inhibitor of metalloproteinase.

In support of our hypothesis that the neutrophils are the source of the increased amounts of MMP-9 in ILD in SSc, BALF MMP-9 levels in emphysema and in differing histologic types of idiopathic interstitial pneumonias, have been found complexed with neutrophil lipocalin, indicating neutrophil origin (Finlay 1997, Suga 2000). Moreover, in the study by Suga et al, MMP-9 levels were elevated and correlated with neutrophil number in usual idiopathic pneumonia (UIP). Immunohistochemical staining showed intense MMP-9 expression in alveolar macrophages, neutrophils and regenerating epithelial lining cells in UIP and also faint expression in these cells in non specific interstitial pneumonia (NSIP), which are the most common types of interstitial pneumonia in ILD in SSc (Suga 2000).

The neutrophil attractant chemokine IL-8 and truncated IL-8 seem to be the factors predominantly responsible for inducing the release of stored MMP-9 from the neutrophil, and stimulating further synthesis. Neutrophil MMP-9 release may even be induced by contact with ECM constituents and lipopolysaccaride from bacterial cell walls. Neutrophils may further contribute to the effectiveness of MMP-9 on substrate degradation by secreting elastase, which is known to inactivate bound and unbound TIMP-1 without damaging the MMP-9 structure (Itoh 1995). Moreover, neutrophil MMP-9 synthesis and release have not been reported inducible by inflammatory cytokines. Thus the concept that Th1 cytokines stimulate, while Th2 cytokines inhibit MMP-9 production does probably not apply to the richest source: the neutrophil, but only to lymphocytes, monocytes/macrophages, possibly even to dendritic cells. Although the T cell reaction in ILD in SSc has been shown to be of a mixed Th1/Th2 type (Majumdar 1999) or of the Th2 type in patients with rapidly progressive lung disease (Atamas 1999), theoretically, the direction of the T cell response should have only minor effects on the amounts of MMP-9 present in the lung tissue.
Recent evidence indicates that MMP-9 is important in the balance between the Th1 and the Th2 reaction, thus in a work by McMillan et al 2004, allergen-challenged MMP-9 knockout mice were shown to develop ILD with massive eosinophil invasion and production of Th2 cytokines by invading Th cells. The mechanism behind this reaction is unknown (McMillan 2004).

Even eosinophils may secrete MMP-9 (Ohno 1997) in minor amounts and we found slight correlations between eosinophil count and levels of total-, pro- and calculated active MMP-9 as well as to the ratios between pro- and active MMP-9 and TIMP-1 (table 4). In the study by Suga et al however, no correlation between eosinophil percentage and MMP-9 levels was found (Suga 2000).

### Table 4

<table>
<thead>
<tr>
<th></th>
<th>rs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total MMP-9</td>
<td>0.54</td>
<td>0.044</td>
</tr>
<tr>
<td>Pro-MMP-9</td>
<td>0.46</td>
<td>0.088</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>0.54</td>
<td>0.042</td>
</tr>
<tr>
<td>Pro MMP-9/TIMP-1</td>
<td>0.686</td>
<td>0.010</td>
</tr>
<tr>
<td>Active MMP-9/TIMP-1</td>
<td>0.606</td>
<td>0.023</td>
</tr>
</tbody>
</table>

The mast cell is yet another cell type producing MMP-9 as well as TIMP-1 (Fang 1999, Frank 2001). In our study the number of mast cells was increased in BALF from SSc patients with ILD as well as in SSc patients with normal HRCT. We found a slight correlation of mast cell count to TIMP-1 levels, but no correlation to MMP-9 levels, indicating that mast cells may be involved predominantly in the healing process and probably also in the process of fibrous scarring in ILD in SSc (table 5). This assumption is based on the findings of Kikuchi et al 1997 that TIMP-1 has a mitogenic effect on SSc fibroblasts and the findings that even other mast cell products such as histamine, chymase and tryptase stimulate fibroblast proliferation and collagen production (Garbuzenko 2002, 2004). The mast cell may, however, play a dual role in tissue remodelling in ILD as α-chymase may activate pro-MMP-9 unbound and complexed with TIMP-1. Furthermore, unbound TIMP-1 and TIMP-1 complexed with MMP-9 and lipocalin is also susceptible to proteolysis by α-chymase (Frank 2001).

### Table 5

<table>
<thead>
<tr>
<th></th>
<th>rs</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>0.52</td>
<td>0.051</td>
</tr>
<tr>
<td>Total MMP-9</td>
<td>0.32</td>
<td>0.23</td>
</tr>
<tr>
<td>Pro-MMP-9</td>
<td>0.34</td>
<td>0.21</td>
</tr>
<tr>
<td>Active MMP-9</td>
<td>0.03</td>
<td>0.90</td>
</tr>
<tr>
<td>Pro MMP-9/TIMP-1</td>
<td>-0.28</td>
<td>0.30</td>
</tr>
<tr>
<td>Active MMP-9/TIMP-1</td>
<td>0.03</td>
<td>0.90</td>
</tr>
</tbody>
</table>

PAPER IV

The bronchial epithelium has to the best of my knowledge not been examined in SSc and thus our results cannot be directly compared to the results of other investigations. In short, we found neutrophil invasion of the bronchial submucosa in SSc but could not pinpoint the mode of recruitment. Thus the bronchial epithelium was inert or depressed and did not express increased levels of CXC chemokines essential to neutrophil homing: IL-8, ENA-78 or GRO-α, nor was there any rise in epithelial expression of inflammatory- and toxin sensitive transcription factors or their nuclear translocation.

On the opposite: IL-8 expression and the expression and nuclear translocation of NFκB were decreased in SSc bronchial epithelium compared to age- and sex matched healthy controls. The number of submucosal CD3+ T lymphocytes, CD4+ Th cells and CD8+ Tc cells as well as mast cell number was similar in SSc patients and controls. In the bronchial submucosa in SSc, vessels expressed adhesion molecules P-selectin and ICAM-1 to the same degree as in healthy controls (table 3 and 4).
Table 3.
Comparison of cytokine expression in the bronchial epithelium, inflammatory cells in the epithelium and submucosa, and endothelial adhesion molecules, between SSc patients and age- and sex matched healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SSc patients</th>
<th>p*</th>
<th>SSc patients (smokers and corticosteroid treated patients excluded)</th>
<th>p*</th>
</tr>
</thead>
<tbody>
<tr>
<td>Neutrophils**</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.002</td>
<td>74.5 (27.9-102)</td>
<td>0.042</td>
</tr>
<tr>
<td>(positively stained cells/mm² submucosa)</td>
<td>35.3 (18.3-49.7)</td>
<td>73.4 (42.1-103)</td>
<td></td>
<td>38.8 (17.7-56.4)</td>
<td>0.16</td>
</tr>
<tr>
<td>Mast cells**</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.10</td>
<td>28.0 (20.5-42.3)</td>
<td>0.29</td>
</tr>
<tr>
<td>(positively stained % positive vessels compared with EN4 staining)</td>
<td>25.8 (15.2-35.6)</td>
<td>20.1 (21.3-56.5)</td>
<td></td>
<td>5.65 (0.00-12.9)</td>
<td>0.69</td>
</tr>
<tr>
<td>CD3a**</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.71</td>
<td>18.0 (8.8-31.1)</td>
<td>0.36</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>16.9 (5.65-42.5)</td>
<td>20.8 (8.90-30.4)</td>
<td></td>
<td>5.70 (0.00-8.60)</td>
<td>0.69</td>
</tr>
<tr>
<td>CD4a**</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.31</td>
<td>25.0 (7.83-39.5)</td>
<td>0.48</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>2.8 (0.96-11.4)</td>
<td>2.10 (0.00-8.60)</td>
<td></td>
<td>5.65 (0.00-12.9)</td>
<td>0.69</td>
</tr>
<tr>
<td>CDS8a**</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.73</td>
<td>18.0 (8.8-31.1)</td>
<td>0.36</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>11.0 (5.90-26.9)</td>
<td>9.70 (3.60-24.3)</td>
<td></td>
<td>5.65 (0.00-12.9)</td>
<td>0.69</td>
</tr>
<tr>
<td>P-selectin % b</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.73</td>
<td>25.0 (7.83-39.5)</td>
<td>0.48</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>16.7 (3.75-34.3)</td>
<td>19.5 (7.10-29.7)</td>
<td></td>
<td>5.70 (0.00-8.60)</td>
<td>0.69</td>
</tr>
<tr>
<td>ICAM-1 % b</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.52</td>
<td>38.0 (17.2-59.2)</td>
<td>0.64</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>32.7 (21.1-47.2)</td>
<td>30.0 (8.30-51.7)</td>
<td></td>
<td>5.02 (0.00-9.2)</td>
<td>0.64</td>
</tr>
<tr>
<td>IL-8 e</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.031</td>
<td>0.33 (0.08-0.59)</td>
<td>0.035</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>1.0 (0.38-1.48)</td>
<td>0.40 (0.03-0.78)</td>
<td></td>
<td>0.02 (0.00-0.09)</td>
<td>0.64</td>
</tr>
<tr>
<td>Gro-α g</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.95</td>
<td>0.01 (0.00-0.06)</td>
<td>0.64</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>0.02 (0.00-0.12)</td>
<td>0.02 (0.00-0.09)</td>
<td></td>
<td>0.02 (0.00-0.09)</td>
<td>0.64</td>
</tr>
<tr>
<td>ENA-78g</td>
<td>N = 17</td>
<td>N=23</td>
<td>0.39</td>
<td>0.15 (0.03-0.30)</td>
<td>0.16</td>
</tr>
<tr>
<td>(positively stained % of epithelial surface with positive staining)</td>
<td>0.24 (0.08-0.79)</td>
<td>0.17 (0.07-0.38)</td>
<td></td>
<td>0.03 (0.00-0.09)</td>
<td>0.64</td>
</tr>
</tbody>
</table>

p*: refers to comparison with healthy controls
a: % positive stained vessels compared with EN4 staining
b: % positive stained vessels compared with EN4 staining
c: % of epithelial surface with positive staining
Data are given as medians with 25th and 75th percentiles

However, submucosal neutrophil invasion correlated inversely and significantly with TLC in patients with ILD. Yet, we could not show increased neutrophil invasion in patients with signs of ILD on HRCT, compared to SSc patients without such signs. The discrepancy may be due to the fact that some patients with ILD had HRCT changes signifying only slight pulmonary engagement. Taken together, our findings may point at a bronchial submucosal involvement in ILD in SSc, possibly with the same etiology as the pulmonary involvement. Thus the bronchial epithelium seems not to be involved in SSc as it is in for example subjects exposed to air pollutants or in patients with asthma, where the primary event in neutrophil and eosinophil recruitment to the submucosa is chemokine synthesis in the epithelium due to activation by for example diesel exhaust or adhering allergens, respectively (Pourazar 2005, Brown 1998).

It is rather well-known that toxic (Bosson 2003) and inflammatory signals (Walz 1991) sent to the bronchial epithelium may increase its expression of IL-8, GRO-α and ENA-78, which are all CXC chemokines with an glu-leu-arg (ELR) motif in the amino-terminal portion essential to neutrophil attraction and activation. GRO-α and IL-8 may furthermore cause changes in motility and shape (increase chemotaxis) in the neutrophil (Xythalis 2002) and delay neutrophil apoptosis (Glynn 2001). Moreover, all three chemokines may via binding to the neutrophil CXCR1 and 2 cause exocytosis of elastase and a respiratory burst (Geiser 1993). We searched the bronchial epithelium for their expression, but could not show any increase in SSc. This finding points at an inert bronchial epithelium in SSc not affected by inflammatory signals or toxins and not involved in the recruitment of the increased number of neutrophils found in the submucosa. However, it is possible that there might have been increased amounts of these CXC chemokines in the submucosa, which we did not examine.
Table 4. 
Comparison of intracellular signalling molecules in the bronchial epithelium, between SSc patients and age-and sex matched healthy controls.

<table>
<thead>
<tr>
<th></th>
<th>Controls</th>
<th>SSc patients</th>
<th>p*</th>
<th>SSc patients</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>N = 17</td>
<td>N=23</td>
<td>(smokers and corticosteroid treated patients excluded)</td>
<td>p*</td>
</tr>
<tr>
<td>ERK tot d</td>
<td>0.50 (0.12-1.90)</td>
<td>0.13 (0.03-0.71)</td>
<td>0.08</td>
<td>0.00 (0.03-0.83)</td>
</tr>
<tr>
<td>ERK nucl *</td>
<td>1338 (589-2301)</td>
<td>705 (165-2052)</td>
<td>0.09</td>
<td>506 (135-1846)</td>
</tr>
<tr>
<td>JNK tot d</td>
<td>0.40 (0.16-0.65)</td>
<td>0.14 (0.09-0.34)</td>
<td>0.08</td>
<td>0.14 (0.10-0.58)</td>
</tr>
<tr>
<td>JNK nucl *</td>
<td>1063 (548-1282)</td>
<td>564 (346-981)</td>
<td>0.06</td>
<td>545 (348-1026)</td>
</tr>
<tr>
<td>p38 tot d</td>
<td>0.04 (0.01-0.11)</td>
<td>0.02 (0.00-0.14)</td>
<td>0.06</td>
<td>0.01 (0.00-0.04)</td>
</tr>
<tr>
<td>p38 nucl *</td>
<td>161 (63-651)</td>
<td>143 (26-799)</td>
<td>0.73</td>
<td>91.6 (29.8-216)</td>
</tr>
<tr>
<td>NFκB tot d</td>
<td>0.06 (0.02-0.31)</td>
<td>0.02 (0.01-0.05)</td>
<td>0.012</td>
<td>0.02 (0.00-0.06)</td>
</tr>
<tr>
<td>NFκB nucl *</td>
<td>440 (186-1235)</td>
<td>169 (47.1-413)</td>
<td>0.006</td>
<td>72.9 (36.6-415)</td>
</tr>
<tr>
<td>c-Fos tot d</td>
<td>0.05 (0.02-0.07)</td>
<td>0.02 (0.01-0.07)</td>
<td>0.21</td>
<td>0.02 (0.01-0.07)</td>
</tr>
<tr>
<td>c-Fos nucl *</td>
<td>309 (132-498)</td>
<td>147 (56.3-340)</td>
<td>0.11</td>
<td>85.2 (36.3-330)</td>
</tr>
<tr>
<td>c-Jun tot d</td>
<td>0.64 (0.39-1.18)</td>
<td>0.59 (0.20-1.42)</td>
<td>0.65</td>
<td>0.37 (0.16-0.70)</td>
</tr>
<tr>
<td>c-Jun nucl *</td>
<td>1516 (1087-2176)</td>
<td>1434 (631-2136)</td>
<td>0.54</td>
<td>1191 (621-1553)</td>
</tr>
</tbody>
</table>

p*: refers to comparison with healthy controls
d:positive cytoplasm and nuclei staining/mm² epithelium
e:numbers of nuclei/mm²

Data are given as medians with 25th and 75th percentiles

Yet, levels of ENA-78 are elevated in BALF from patients with idiopathic ILD (Nakayama 2005), in which disease peripheral blood neutrophils seem primed by IL-8 and GRO-α to easily undergo change in shape and motility upon a second encounter (Glynn 2001). However, in the blood of SSc patients, ENA-78 levels are elevated especially in patients with ILD (Nomura 2008), as are GRO-α levels, which may correlate with decrease in DLco and lung vital capacity (Furuse 2003). Thus there are signs that these chemokines are involved in the tissue destruction in ILD in SSc, however, our findings show that they are not generated in the bronchial epithelium.

Three well-defined subfamilies of mitogen-activated protein kinases (MAPKs) operate in humans. These enzymes are regulated by a characteristic phosphorylation system in which a series of protein kinases phosphorylate and activate one another (figure 1).

The extra-cellular signal-regulated kinases (ERKs) primarily function in the control of cell division and proliferative response to growth factors, while the c-Jun amino-terminal kinases (JNKs) are critical regulators of transcription of several cytokine genes in response to environmental stress and growth factors. P38 MAPKs control the transcription of several cytokines and are activated by inflammatory cytokines such as TNFα and may contribute to allergic and autoimmune diseases. The control of cytokine synthesis and signalling through MAPKs is complex, for example IL-8 transcription and protein synthesis in human airway epithelial cells in response to allergen exposure is mediated via all three MAPK pathways: JNK, ERK and p38, but also via activation of NFkB (Kim 2006, Mitjans 2008).
Figure 1. MAPK phosphorelay systems

MAPKs are part of a phosphorelay system composed of three sequentially activated kinases, and, like their substrates, MAPKs are regulated by phosphorylation. MAPKs serve as phosphorylation substrates for MAPK kinases (MKKs). MKK catalyzed phosphorylation activates the MAPK and increases its activity in catalyzing the phosphorylation of its own substrates. MAPK phosphatases reverse the phosphorylation and return the MAPK into the inactive state. MKKs are highly specific as are the MAPK kinase kinases (MKKKs), which phosphorylate and activate specific MKKs. The different MKKKs can be matched with specific MKK-MAPK cassettes, such that cells can respond to a stimulus with the activation of a specific MAPK pathway.


Our finding of a normal expression of all three subclasses of MAPKs in the bronchial epithelium in SSc points in the same direction as the finding of normal amounts of ENA-78 and GRO-α and low levels of IL-8, that is at an inert or depressed epithelium. We did not examine the submucosa or the endothelium for MAPKs.

The transcription factor NFκB is present in the cytoplasm in complex with inhibitory KB (IKB). Upon stimulation with inflammatory cytokines like IL-1β, IL-6 and TNFα IKB is phosphorylated by inhibitory KB kinases (IKKs), ubiquinated and degraded, releasing nuclear localisation sequences on NFκB, which is then exported to the nucleus, where it may initiate the transcription of a long array of substances important to the inflammatory reaction like adhesion molecules: E-selectin, P-selectin, VCAM-1 and ICAM-1, iNOS, metalloproteinases, cyclo-oxygenase 2, pro-inflammatory cytokines (IL-1β, IL-6, TNFα and IL-2) and chemokines (IL-8, ENA-78, GRO-α). Specific inhibition of NFκB has been shown to inhibit inflammatory diseases in animal models, for example eosinophilic airway inflammation (Verma 2004, review). We examined the bronchial epithelium in SSc for the presence of NFκB in the cytoplasm as well as in the nuclei and found decreased cytoplasmic staining for NFκB as well as a decreased number of cell nuclei harbouring the transcription factor. These findings furthermore strengthen the concept of an inert or depressed bronchial epithelium in SSc.

Furthermore, we stained the vessels of the submucosa for the presence of adhesion molecules: P-selectin and ICAM-1 and found normal expression in SSc with and without ILD. Thus this examination did not point out the pathway of recruitment of neutrophils to the submucosa. It has previously been shown that SSc patients with ILD have increased levels of sE-selectin in their BALF but normal levels of ICAM-1 (Southcott 1998). Moreover, it has been shown that alveolar epithelial lining cells express ICAM-1 constitutively and may increase the expression in regions with idiopathic ILD, in which disease ICAM-1 is expressed on small vessels in areas with alveolitis (Nakao 1995). E-selectin
expression is normal in ILD in SSc and is found on and around moderate to large blood vessels. Nor does the expression of ICAM-1 and VCAM-1 in ILD in SSc differ from the expression in normal lung tissue (Southcott 1998).

Despite this, it may be possible that other adhesion molecules than ICAM-1 and P-selectin, such as E-selectin and VCAM-1 are upregulated in the submucosal vessels of the bronchial epithelium in SSc, especially as eS-selectin has previously been found increased in BALF in ILD in SSc. This assumption would lend an explanation to the neutrophil infiltration. Yet, this remains to be shown.

**PAPER V**

In this work, we chose to examine T lymphocytes and monocytes/macrophages from blood and BALF for the presence of mRNA for a broad panel of cytokines to be able to discriminate between a Th1 and a Th2 reaction profile in SSc. Also mRNA for IL-10 and TGFβ, characteristic of Treg activity were among the cytokine mRNAs looked for.

We found that alveolar T lymphocytes from SSc patients expressed IL-6, IL-10 and IFNγ mRNA more often than those from healthy controls, independent of the presence of ILD. Messenger RNA for TGFβ was present in all alveolar T cell samples from patients as well as controls (table 1).

**Table 1.** Number of positive RT-PCR on mRNA from alveolar- and peripheral blood CD3+ T lymphocytes (T ly) in 18 SSc patients and 16 age- and sex matched controls.

<table>
<thead>
<tr>
<th>Cytokine</th>
<th>Patients Alveolar T ly</th>
<th>Controls Alveolar T ly</th>
<th>Patients Blood T ly</th>
<th>Controls Blood T ly</th>
</tr>
</thead>
<tbody>
<tr>
<td>IL-1β</td>
<td>13*</td>
<td>7</td>
<td>13***</td>
<td>1</td>
</tr>
<tr>
<td>IL-2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-3</td>
<td>1</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-4</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-5</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-6</td>
<td>16***</td>
<td>3###</td>
<td>16</td>
<td>16</td>
</tr>
<tr>
<td>IL-7</td>
<td>01</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>IL-8</td>
<td>14</td>
<td>14</td>
<td>18</td>
<td>14</td>
</tr>
<tr>
<td>IL-10</td>
<td>14 *** €€€</td>
<td>3</td>
<td>3</td>
<td>4</td>
</tr>
<tr>
<td>IFNγ</td>
<td>11** €</td>
<td>0</td>
<td>16***</td>
<td>3</td>
</tr>
<tr>
<td>TNFα</td>
<td>17</td>
<td>15</td>
<td>18</td>
<td>16</td>
</tr>
<tr>
<td>TNFβ</td>
<td>0 €€€</td>
<td>0</td>
<td>15***</td>
<td>1</td>
</tr>
<tr>
<td>TGFβ</td>
<td>141</td>
<td>16</td>
<td>141</td>
<td>16</td>
</tr>
<tr>
<td>GM-CSF</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>CD45RO</td>
<td>141</td>
<td>16</td>
<td>141</td>
<td>16</td>
</tr>
</tbody>
</table>

IL = interleukin; IFN = interferon; TNF = tumor necrosis factor; TGF = transforming growth factor; GM-CSF = granulocyte-macrophage colony stimulating factor; RT-PCR = reverse transcription polymerase chain reaction.

* P < 0.05; ** P < 0.01; *** P < 0.001 BAL patients compared to BAL controls
€ P < 0.05; €€ P < 0.01; €€€ P < 0.001 BAL patients compared to blood patients
*** P < 0.001 Blood patients compared to blood controls
### P < 0.001 BAL controls compared to blood controls
1: n = 14

Statistics: X² test and Fisher’s exact test

Blood T cells significantly more often expressed IL-1β, TNFβ and IFNγ in SSc patients than in matched controls. Messenger RNA for TGFβ was present in all T cell samples from patients as well as controls. Thus the T cell cytokine profile was mixed Th1/Th2 in the lungs of SSc patients and indicated the
presence of regulatory T cells of both the Tr1 (IL-10 secreting) and Th3 (TGFβ secreting) type. Moreover, SSc alveolar T cells seemed to be locally activated as they harboured IL-10 mRNA more often than peripheral blood T cells. We could show no correlation of the presence of IFNγ mRNA in alveolar T cells to ILD.

There was no correlation between the presence of IFNγ mRNA in peripheral blood T cells and the presence of diffuse skin engagement, nor was there any correlation to disease duration. There were no dominating combinations of cytokine mRNAs. However, the number of patients in our study was limited and it is possible that disease duration and disease activity may play a role in defining the kind of immune reaction mounted.

Both IL-6 and IL-10 are cytokines typical for the Th2 reaction, driving the humoral immune response. Both activate B cells to proliferate and mature into antibody producing plasma cells. Moreover, a Th2 reaction activates and attracts eosinophils and mast cells into the affected organ. We found that eosinophil number was increased in the BALF from SSc patients with ILD and that the number of alveolar mast cells was increased in all SSc patients independent of lung engagement. These findings corroborate the notion of an ongoing Th2 reaction in SSc lung. Others have shown that the Th2 reaction dominates in patients with rapidly progressive ILD, and therefore it has been theorised that a shift to a Th1 reaction, which drives cell-mediated immunity may diminish disease activity and thus be beneficial. We showed that the presence of IFNγ mRNA in alveolar T cells was more common in SSc patients than in healthy controls, pointing at the possibility that SSc patients may be able to mount a defence against the deleterious Th2 reaction themselves. This is in accordance with the finding that ILD is often self-limiting in SSc (Wallaert 1986, Remy-Jardin 1993).

In peripheral blood T cells mRNA for IL-1β, IFNγ and TNFβ were more common in patients than in healthy controls. Thus cytokine transcripts of the Th1 cell reaction dominated, while mRNAs for cytokines of the Th2 reaction (IL-6, IL-10) were found equally often in patients and controls. Our findings are in line with the findings by Gruschwitz 1997, Sato 2000 and Valentini 2001, who also showed Th1 reaction preponderance in PBMC and blood Th cells. Others, however, have found that the Th2 reaction dominate in SSc PBMC (Famularo 1990, Needleman 1992, Hasegawa 1997, Tsuji-Yamada 2001). There are signs that a Th2 reaction may dominate in patients with active disease and diffuse skin engagement (Tsuji-Yamada 2001, Giacomelli 1997). Thus the possibility exists, that the Th1 reaction we found in peripheral T cells, may mirror a defence mechanism mounted by the organism itself against the pathological immune response in SSc.

The finding of TGFβ mRNA in almost all alveolar- and peripheral blood T cell samples from SSc patients and healthy controls point at the likely presence of Treg cells, that is TGFβ secreting Th3 cells, in lungs and blood. Alveolar T cells from SSc patients harboured IL-10 mRNA at a greater frequency than healthy controls. Whether this transcription was produced by Th2 cells or regulatory T cells of the Tr1 type remains to be elucidated. It is not surprising that signs of Treg cell activity were found in the samples as Treg cells comprise about 5-20% of the total T cell number in healthy persons. Treg cells work in order to secure self-tolerance through the suppression of effector Th1 and Th2 cells, and a defect in Treg cell function may be anticipated in an auto-immune disease like SSc. The question of a disturbed Treg function in SSc remains to be elucidated.
CONCLUSIONS

In this study pathogenetic mechanisms of vascular changes and ILD in SSc were investigated.

In the works on vascular and endothelial function in SSc, we found evidence of an increased production of NO correlating to elevated levels of soluble adhesion molecules: E-selectin, VCAM-1 and ICAM-1. The factor analysis showed loading of one factor on all of these four variables, pointing at a common, probably endothelial origin. We propose that NO as well as adhesion molecules may emanate from the activated, chronically inflamed vascular endothelium. Moreover, the urinary excretion of the second downstream messenger of NO stimulation: cGMP is elevated in SSc. A finding, which supports the notion of increased endothelial NO production leading to increased stimulation of VSMC guanylate cyclase.

The stiffness of the wall of the radial artery is increased in SSc due to chronic inflammation. Decreased endothelium-dependent- and independent dilation of the brachial artery could not be shown by high-resolution ultra-sound imaging. However, correlation analysis showed a strong influence of endothelial activation on these two variables, especially on endothelium-independent dilation. Thus these two measures of endothelial and vascular function may be reduced in the brachial artery in SSc, but our study had too little power to show this, either the high-resolution ultrasound method was to crude or the number of patients too small. Moreover, the result of the factor analysis was in agreement with the correlation analysis, and even gave rise to a suspicion of the presence of nitrate tolerance.

In SSc with ILD, levels of total MMP-9, pro-MMP-9 and calculated levels of active MMP-9 in alveolar fluid are increased. Levels of MMP-9 derivatives are probably produced by an increased number of neutrophils invading the alveolar interstitium and to a lesser extent by eosinophils, which is in agreement with known neutrophil- and eosinophil biology. Moreover, levels of TIMP-1, the natural inhibitor of MMP-9, were elevated in both SSc with and without ILD, to which end an elevated number of alveolar mast cells probably contributed. Correlation studies pointed at a role for MMP-9 in the destruction of pulmonary tissue in ILD in SSc, leading to decreased total lung capacity.

The bronchial submucosa is invaded by neutrophils in SSc, but the epithelium is inert without signs of inflammatory cytokine activation of transcription factors or increased nuclear translocation. The pathway leading to neutrophil invasion is uncertain as epithelial expression of neutrophil attracting chemokines is normal or depressed and the submucosal endothelial expression of the examined adhesion molecules is normal. Neutrophil invasion correlates to decrease in lung capacity and thus there may be a common etiology with ILD in SSc.

There is a mixed Th1/Th2 reaction in the alveolar interstitium in SSc and a Th1 reaction in peripheral blood. Alveolar T cells are probably locally activated. We could show no difference between SSc with and without ILD. Treg cells of both the Tr1 and Th3 subset may be present in the lungs as well as in the blood in SSc.
SAMMANFATTNING PÅ SVENSKA

Systemisk skleros (SSc) är en bindvävs inflammatorisk sjukdom, som främst drabbar kvinnor i medelåldern. Första sjukdomsmyndet är oftast Raynaud’s fenomen med vita fingrar vid kyla, blåst och stress. Efter uppkomsten av Raynaud’s fenomen utvecklas hudförändringar med svullnad och stramhet på fingrar och händer med eventuell spridning till större delar av kroppen. Vid utbredda hudförändringar finns det risk för tillkomst av immunologisk inflammation i lungorna, njurengagement med plötsligt stigande blodtryck och nedsättning av njurfunktionen, inflammation och stelhet i magtarmkanalen samt i hjärtmuskeln. Patienter med hudförändringar begränsade till händerna utvecklar ibland kärlförändringar i lungkretslöpet med högt blodtryck efter 10-20 års sjukdom.

Vid SSc har påvisats inflammatoriska förändringar i kärlvägg med förtjockning och nedsättning av tanklig adventit, som orsakar ökad NO-syntes i endotelet. Den framkallade dilatationen är pålitligt mått på stelheten av artär väggen mättes med applanationstonometri. Medelst ultraljud mättes dessa markörer tyder på att denna artär är engage

Inflammationen orsakar den ökade stelheten påkändes i Radialis artärernas vägg och vidgningen av Brachialis artären efter stimulering korrelerade till markörerna för kärlinflammation: nitrat och adhesionsmolekyler, vilket pekar på ett kausalt samband med inflammationen.

I delstudium I fann vi hållpunkter för en ökad syntes av det kärlvidgande ämnet kväveoxid (NO) i cellerna i kärlväggarna (endotelet) vid SSc, samt relatera fyndet till tidigare studier t.ex. att periferiska arterier vid SSc har ökat stelhet och att NO-syntesen ibland försvinner.

I delstudium II fann vi en ökad stelhet av kärlväggen i Radialis artären vid handleden samt normal vidgning av Brachialis artären. Vi mätte blodets och urinens halter av nitrat och nitrit som ett mått på NO-syntesen. Vi mätte blodets och urinens halter av nitrat och nitrit som ett mått på NO-syntesen. Vi mätte blodets och urinens halter av nitrat och nitrit som ett mått på NO-syntesen. Vi mätte blodets och urinens halter av nitrat och nitrit som ett mått på NO-syntesen.
(NTG%) eller endotel oavhängig dilatation. Vid strukturella förändringarna av kärlväggen med ökad stelhet är NTG% nedsatt.

I delstudium III fann vi att lungornas vita blokdroppar vid ILD utsöndrar ökade mängder av det vävnadsbedbytande ämnet metalloproteinase 9 (MMP-9) samt av dess hämmer: inhibitor of metalloproteinase 1 (TIMP-1). MMP-9 bildas av leukocytter i syfte att bryta ner hinder under deras vandring i olika organ och vävnader och har förmağan att bryta ner kollagen och elastin, som även skiljeväggarna mellan lungblåsorerna (alveolerna) till stor del består av. Vi mätte halterna i vätska från alveolerna hos 9 SSc patienter med ILD, 7 SSC patienter med normala lungor och friska kontroller och fann att de förhöjda halterna av MMP-9 korrelerade till graden av lungfunktions nedsättning vid ILD. Detta tyder på ett patogenetiskt samband. MMP-9 bildas främst av den såkallade neutrofilla leukocytten i syfte att bryta ned vävnader vid bekämpning av svårtillgängliga bakteriella infektioner. Vi fann även ökade mängder myeloperoxidas (MPO), som också utsöndras av neutrofilla leukocytter samt en korrelation mellan antalet neutrofilla leukocytter och mängden MMP-9 och MPO i vätskan.

TIMP-1 halter var ökade i vätskan från alveolerna hos SSC patienter såväl med som utan ILD. TIMP-1 bildas också av leukocytter och fyndet kan tyda på att dessa är aktiverade i lungorna vid SSc trots att förändringar inte går att se med röntgen. Vi mätte antalet neutrofila leukocytter och korrelerade till minskningen av lungfunktionen hos patienter med ILD genom att bilda TIMP-1, som hämmer MMP-9s vävnadsbedbytande effekt.

I delstudium IV fann vi att slemhinnan i bronkerna hos SSc patienter innehåller ett ökat antal neutrofilla leukocytter. Dessutom att det yttersta lagret av celler (epitelet) inte är aktiverat vid SSc, i motsättning till fyndet vid asthma. Bronkslemhinna hos 23 patienter med SSC, varav 12 med ILD färgades immunohistokemiskt för närvaro av neutrofilla leukocytter. Bronkslemhinna hos 9 SSc patienter med ILD, 7 SSC patienter med normala lungor och friska kontroller och fann att de förhöjda halterna av MMP-9 korrelerade till graden av lungfunktions nedsättning vid ILD. Detta tyder på ett patogenetiskt samband. MMP-9 bildas främst av den såkallade neutrofilla leukocytten i syfte att bryta ned vävnader vid bekämpning av svårtillgängliga bakteriella infektioner. Vi fann även ökade mängder myeloperoxidas (MPO), som också utsöndras av neutrofilla leukocytter samt en korrelation mellan antalet neutrofilla leukocytter och mängden MMP-9 och MPO i vätskan.

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I delstudium V fann vi att lymfocytter från SSc patienters lungblåsor är aktiverade och bildar ämnen (interleukiner (IL)), som även bildas vid olika typer av infektioner, allergiska och autoimmana sjukdomar. Vid undersöknin av lymfocytter från alveolar och blod, fann vi en ökad förekomst av IL-6 i alveolära lymfocytter, en ökad förekomst av interferon (IFN)γ i såväl alveolära som bloplasma leukocytter och en ökad förekomst av IL-10 i alveolära lymfocytter från SSc patienter. Alveolära lymfocytter från SSc patienter med svår ILD bildar interleukiner, som vid en allergisk reaktion: IL-4,5,6 och 10, medan alveolära lymfocytter från SSc utan ILD bildar IFNγ. Våra fynd visade en bland reaktion, dvs. som man skulle kunna hitta hos patienter med tendens till ILD utveckling, men där kroppens försvar klarar av att stävja denna medelst en motreaktion. Självlimiterande ILD är vanligt förekommande vid SSc, våra fynd kan även tala för detta.

Sammanfattningsvis visade studierna i avhandlingen att NO produktionen är ökad vid ILD och SSc korrelerar till markörer för kärlinflammation, talande för NO produktionen är ökad i endotelcellerna. Dessutom fann vi en ökad stelhet av kärlväggen i Radialis artären sannolikt till följd av inflammation. Men med den tillgängliga ultraljudsmetoden lyckades vi inte visa en nedsatt endotel oavhängig dilatation av Brachialis artären, dock tyder resultatet av korrelationsanalysen på att båda former för dilatation är begränsade av kärlengagemanget (inflammationen), dvs. att endotel funktionen är påverkat och att det finns strukturella kärförändringar. Korrelationsanalyserna och faktoranalysen pekade på förekomst av nitrat tolerans. Vi fann ökade halter av MMP-9, TIMP-1 och MPO i alveolerna hos SSc patienter med ILD. Halterna av MMP-9 korrelerade till graden av lungfunktions nedsättning, tydande på att MMP-9 ansvarar för nedbrytningen av lungvävnad vid ILD. Halten av MMP-9 och MPO korrelerade till antalet neutrofilla leukocytter, vilket pekar på dessa som ursprung till enzymerna. Antalet neutrofilla leukocytter är ökat i bronkslemhinna och korrelerar till graden av lung funktions nedsättning vid ILD, tydande på gemensam patogenes. Bronkepitelet är inert med låga halter av inflammations-aktiverade transkriptionsfaktorer, och neutrofilla leukocytter är ökat i bronkslemhinna och korrelerar till graden av lung funktions nedsättning vid ILD, tydande på gemensam patogenes. Bronkepitelet är inert med låga halter av inflammations-aktiverade transkriptionsfaktorer, och neutrofilla leukocytter är ökat i bronkslemhinna och korrelerar till graden av lung funktions nedsättning vid ILD, tydande på gemensam patogenes. Bronkepitelet är inert med låga halter av inflammations-aktiverade transkriptionsfaktorer, och neutrofilla leukocytter är ökat i bronkslemhinna och korrelerar till graden av lung funktions nedsättning vid ILD, tydande på gemensam patogenes.
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