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Systemic sclerosis

Novel molecular and epidemiological features of disease

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AKADEMISK AVHANDLING

som för avläggande av doktorsexamen i medicinsk vetenskap vid medicinska
fakulteten vid Lunds universitet

offentligen kommer att försvaras fredagen den 20 december 2013

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Professor Dr. Ulf Müller-Ladner

Justus-Liebig-Universität Gießen, Tyskland

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<p>Abstract</p> <p>Systemic sclerosis (SSc) is a systemic rheumatic disease with significant mortality and morbidity. Different estimations of disease prevalence and incidence have been presented from various parts of the world. While lung involvement is a common cause of SSc-related death, a majority of SSc patients suffer from symptoms originating in the gastrointestinal (GI) tract.</p> <p>By combining data from a population based register with individual case ascertainment, we investigated the epidemiology of SSc in a defined region in southern Sweden comprising one million adult inhabitants. The investigation was performed by using classification criteria presented in 1980 and in 2013 respectively. SSc prevalence was estimated to 305 per million and the annual incidence 19 per million and year by application of the 2013 classification criteria. We show that the novel criteria identify SSc subjects who were overlooked by the previous criteria. Usage of either criteria set resulted in prevalence estimates that are higher than previous reports from northern Europe, but similar to reports from southern Europe.</p> <p>Cartilage oligomeric matrix protein (COMP) is a promising biomarker of skin fibrosis in SSc. We have investigated the potential for S-COMP to serve as a biomarker for SSc associated lung fibrosis and as a predictor of SSc survival. S-COMP showed only minimal associations with the development or presence of pulmonary fibrosis. SSc patients with pathological S-COMP in early SSc were at an increased risk of death. To further explore the mechanisms behind COMP and fibrosis, we investigated skin fibrosis in mice deficient in COMP. These mice were not resistant to skin fibrosis.</p> <p>GI disease is a common visceral manifestation of SSc. The inflammatory protein complex S100A8/A9, also known as calprotectin, has been associated with several rheumatic diseases. Faecal calprotectin (FC) is a validated biomarker in inflammatory bowel disease. We have explored the biomarker potential of FC in SSc. FC correlated with SSc manifestations in the GI tract. FCs showed little variation upon repeated testing. FC was higher in SSc compared to other rheumatic diseases.</p> <p>The development of inflammation and fibrosis was investigated in reference to S100A8/A9 in an experimental mouse model. S100A8 and S100A9 were found to localise to inflamed and fibrotic skin tissue. Using the same model, we could not identify any significant reduction of inflammation or fibrosis in S100A9 deficient mice.</p> <p>I suggest that FC, a feasible biomarker already available in routine clinical care, could be a valid biomarker of GI disease in SSc. Further studies are warranted to elucidate the mechanisms behind pathological FC testing in SSc.</p>		
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Systemic sclerosis

Novel molecular and epidemiological features of disease

Kristofer Andréasson



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Lund University
2013

The symbol on the front page with the willow trees and the colours of the Scandinavian flags was developed for the Scandinavian Congress of Rheumatology arranged in Malmö in 1992.

It has since then been the symbol of The Department of Rheumatology in Lund. In this thesis discoveries made some decades ago in Denmark, Norway and Sweden are discussed.

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List of papers

This thesis is based on the following communications:

1. Andréasson K., Saxne T., Bergknut C., Hesselstrand R., Englund M.: Prevalence and incidence of systemic sclerosis in southern Sweden: population-based data with case ascertainment using the 1980 ARA criteria and the proposed ACR-EULAR classification criteria. *Ann Rheum Dis*, [epub ahead of print] Jul 29. doi:10.1136/annrheumdis-2013-203618
2. Hesselstrand R., Andréasson K., Wuttge DM., Bozovic G., Scheja A., Saxne T.: Increased serum COMP predicts mortality in SSc: results from a longitudinal study of interstitial lung disease. *Rheumatology (Oxford)* 2012;**51**(5):915-20
3. Andréasson K., Scheja A., Saxne T., Ohlsson B., Hesselstrand R.: Faecal calprotectin: a biomarker of gastrointestinal disease in systemic sclerosis. *J Intern Med* 2011;**270**(1):50-57
4. Andréasson K., Saxne T., Scheja A., Bartosik I., Mandl T., Hesselstrand R.: Faecal levels of calprotectin in systemic sclerosis are stable over time and are higher compared to primary Sjögren's syndrome and rheumatoid arthritis. *Submitted manuscript under revision*.
5. Andréasson K., Gustafsson R., Roth T., Vogl J., Ivars F., Hesselstrand R., Saxne T.: S100A8/A9, a damage associated molecular pattern (DAMP) protein, is not essential for the development of inflammatory associated skin fibrosis in mouse. *Manuscript*.

Some additional observations not previously presented have been included in the Results and Discussion sections of this thesis.

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I have also contributed to the following communications, prepared during my Ph.D. studies, not included in the thesis:

Rydell-Törmänen K., Andréasson K., Hesselstrand R., Risteli J., Heinegård D., Saxne T., Westergren-Thorsson G.: Extracellular matrix alterations and acute inflammation; developing in parallel during early induction of pulmonary fibrosis. *Lab Invest* 2010;**92**(6):917-25

Hesselstrand R., Wildt M., Bozovic G., Andersson-Sjöland A., Andréasson K., Scheja A., Westergren-Thorsson G., Bjermer L., Wuttge DM.: Biomarkers from bronchoalveolar lavage fluid in systemic sclerosis patients with interstitial lung disease relate to severity of lung fibrosis. *Respir Med* 2013;**107**(7):1079-86

Agarwal P., Schulz JN., Blumbach K., Andréasson K., Heinegård D., Paulsson M., Mauch C., Eming SA., Eckes B., Krieg T.: Enhanced deposition of cartilage oligomeric matrix protein is a common feature in fibrotic skin pathologies. *Matrix Biol* 2013;**32**(6):325-31

Abbreviations

ACR	American College of Rheumatology
ARA	American Rheumatology Association
BLM	Bleomycin
COMP	Cartilage oligomeric matrix protein
CTGF	Connective tissue growth factor
DAMP	Damage associated molecular pattern molecule
dcSSc	Diffuse cutaneous SSc
ELISA	Enzyme-linked immunosorbent assay
EULAR	European League Against Rheumatism
FC	Faecal calprotectin
GAVE	Gastric antral vascular ectasia
GI	Gastrointestinal
HRCT	High-resolution computed tomography
ILD	Interstitial lung disease
lcSSc	Limited cutaneous SSc
lSSc	Limited SSc
mRSS	Modified Rodnan Skin Score
OA	Osteoarthritis
PAH	Pulmonary arterial hypertension
PDGF	Platelet-derived growth factor
PPI	Proton pump inhibitor
pSS	Primary Sjögren's syndrome
RA	Rheumatoid arthritis
RAGE	Receptor for advanced glycation end products
SSc	Systemic sclerosis
TGF- β	Transforming growth factor beta
TLR	Toll-like receptor
TSP	Thrombospondin

Introduction

This thesis concerns systemic sclerosis (SSc), a systemic rheumatic disease with significant mortality and morbidity. I have investigated this disease with reference to:

- a) novel and classical classification criteria for SSc
- b) a biomarker measurable in serum: cartilage oligomeric matrix protein (COMP)
- c) a biomarker measurable in faeces: faecal calprotectin (FC)

In this introductory chapter, it is my intention to present and discuss some current knowledge in these and neighbouring fields in order to put the novel data presented in this thesis in an appropriate context.

Systemic sclerosis

Diagnosis and classification

SSc is an autoimmune connective tissue disease that is relatively uncommon and also heterogeneous in the way it presents in different patients. The clinical rationale for including various combinations of clinical manifestations of SSc within one common term can be, and has been, questioned.[1]

Different subtypes of SSc have been suggested during the last decades. There is a general agreement that the subdivision of SSc in diffuse SSc (dSSc) and limited SSc (lSSc) is not only clinically relevant and helpful, but also relatively easy to apply in routine clinical care.[1, 2] Importantly, the clinical differences between these subtypes have been shown to have molecular and immunological correlates. The term dSSc was introduced in 1988 and refers to patients suffering from skin fibrosis both proximal and distal to the elbows and knees. These patients often have visceral manifestations of SSc and have a substantially increased mortality rate.[3, 4] Patients with lSSc do not suffer from extended skin fibrosis and have clinically (yet not necessarily molecular) normal skin proximal to the elbows and knees. These patients are less often subject to some visceral manifestations of SSc

but are still at high risk of eventually developing pulmonary arterial hypertension (PAH) and digital ulcers.[2, 5] A subgroup of lSSc named systemic sclerosis sine scleroderma, has been proposed. This subgroup refers to patients satisfying any SSc criteria without presenting actual skin fibrosis. A consequence of recognising this subgroup has been to divide SSc into diffuse cutaneous SSc (dcSSc), limited cutaneous SSc (lcSSc) and limited SSc (lSSc).[6] The clinical and/or scientific value of this further subclassification remains to be explored. In this thesis I will use the classification of lSSc and dSSc as presented in 1988.[2]

The combination of calcinosis, Raynaud's phenomenon, oesophageal dysfunction, sclerodactyly and telangiectasia as a possible subtype of lSSc was suggested already in 1959.[7] The acronym CREST was later introduced.[8] This term is not well defined and the scientific or clinical value of its usage rather limited, and some authorities recommend to abandon it altogether.[9] Another term of questionable value is progressive SSc, which in many cases has been used in reference to dSSc. Several manifestations of lSSc, not at least fatal PAH, are progressive over time, and skin manifestations of dSSc can sometimes regress. Because of this, progressive SSc has been regarded as a confusing and inappropriate term.[2]

Overlap syndrome is a term that has been proposed for patients with SSc who also fulfil criteria for other rheumatic diseases. The rationale for this subgroup may be dubious because of the wide heterogeneity of clinical features expressed by different overlap patients.[10] For example, a SSc patient with erosive rheumatoid arthritis may have few, if any, clinical symptoms in common with a SSc-patient with co-existent polymyositis or systemic lupus erythematosus.

The 1980 ARA (American Rheumatology Association) preliminary classification criteria of SSc has for many years been gold standard in the field of SSc research (table 1).[1, 10, 11] These criteria were selected in order to correctly classify patients with established SSc.[10] They were an important contribution in the field of SSc but they remained preliminary. For many years, additive studies have shown that the 1980 ARA criteria fail to recognise early SSc and do not correctly classify patients with limited skin manifestations of disease.[12]

In October 2013, a joint committee representing the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) published the 2013 classification criteria for SSc (table 2 with definitions presented in table 3). These criteria were first presented at the ACR meeting in Washington D.C. 2012.[13] They were developed with an intention to allow inclusion of early forms of the disease without frank cutaneous sclerosis.[11]

Table 1. 1980 ARA preliminary classification criteria for SSc[10]

Major Criterion
1. Scleroderma* proximal to the metacarpophalangeal joints
Minor Criteria
1. Scleroderma* limited to the fingers (sclerodactyly)
2. Digital pitting scars of fingertips or loss of substance of the distal finger pad†
3. Bibasilar pulmonary fibrosis
Exclusion criteria
Localised scleroderma and pseudosclerodermatous disorders
One major or two minor criteria are considered necessary to classify a patient as SSc

* typical sclerodermatous skin changes: tightness, thickening, and non-pitting induration

† defined as: depressed areas at tips of digits or loss of digital pad tissue as a result of digital ischaemia rather than trauma or exogenous causes

Table 2. The ACR/EULAR criteria for the classification of systemic sclerosis[11]

Item	Sub-item(s)	Weight/score
Skin thickening of the fingers of both hands extending proximal to the metacarpophalangeal joints (<i>sufficient criterion</i>)	-	9
Skin thickening of the fingers (<i>only count the higher score</i>)	Puffy fingers	2
	Sclerodactyly of the fingers (distal to the metacarpophalangeal joints but proximal to the proximal interphalangeal joints)	4
Fingertip lesions (<i>only count the higher score</i>)	Digital tip ulcers	2
	Fingertip pitting scars	3
Telangiectasia	-	2
Abnormal nailfold capillaries	-	2
Pulmonary arterial hypertension and/or interstitial lung disease (<i>maximum score is 2</i>)	Pulmonary arterial hypertension	2
	Interstitial lung disease	2
Raynaud's phenomenon		2
SSc-related autoantibodies (anticentromere, anti-topoisomerase I [anti-Scl-70], anti-RNA polymerase III) (<i>maximum score is 3</i>)	Anticentromere	3
	Anti-topoisomeras I	
	Anti-RNA polymeras III	

Exclusion criteria: Any patient with skin thickening sparing the fingers and patients with scleroderma-like disorder that better explains their manifestations.

The total score is determined by adding the maximum weight in each category. Patients with a total score of 9 or above are classified as SSc.

Table 3. Definitions of items/sub-items in the ACR/EULAR criteria for the classification of systemic sclerosis[11]

Item	Definition
Skin thickening	Skin thickening or hardening not due to scarring after injury, trauma, etc.
Puffy fingers	Swollen digits - a diffuse, usually nonpitting increase in soft tissue mass of the digits extending beyond the normal confines of the joint capsule. Normal digits are tapered distally with the tissues following the contours for the digital bone and joint structures. Swelling of the digits obliterates these contours. Not due to other causes such as inflammatory dactylitis.
Fingertip ulcers or pitting scars	Ulcers or scars distal to or at the proximal interphalangeal joint not thought to be due to trauma. Digital pitting scars are depressed areas at digital tips as a result of ischaemia, rather than trauma or exogenous causes.
Telangiectasia	Telangiectasiae are visible macular dilated superficial blood vessels, which collapse upon pressure and fill slowly when pressure is released. Telangiectasiae in a scleroderma-like pattern are round and well demarcated and found on hands, lips, inside of the mouth, and/or are large mat-like telangiectasiae. Distinguishable from rapidly filling spider angiomas with central arteriole and from dilated superficial vessels.
Abnormal nailfold capillary pattern consistent with systemic sclerosis	Enlarged capillaries and/or capillary loss with or without pericapillary haemorrhages at the nailfold. May also be seen on the cuticle.
Pulmonary arterial hypertension	Pulmonary arterial hypertension diagnosed by right-sided heart catheterisation according to standard definitions.
Interstitial lung disease	Pulmonary fibrosis seen on high-resolution CT or chest radiography, most pronounced in the basilar portions of the lungs, or occurrence of 'Velcro' crackles on auscultation, not due to another cause such as congestive heart failure.
Raynaud's phenomenon	Self-reported or reported by a physician, with at least a 2-phase colour change in finger(s) and often toe(s) consisting of pallor, cyanosis, and/or reactive hyperemia in response to cold exposure or emotion; usually one phase is pallor.
SSc-related auto-antibodies	Anticentromere antibody or centromere pattern seen on antinuclear antibody testing, anti-topoisomerase I antibody (also known as anti-Scl-70 antibody), or anti-RNA polymerase III antibody. Positive according to local laboratory standards.

Epidemiology

Estimation of prevalence and incidence of rare diseases is problematic, especially when correct diagnosis may demand referral to a specialist centre.[14] Different estimations of SSc prevalence in various parts of the world have been presented. The majority of these studies have applied the 1980 ARA classification criteria.[15]

SSc prevalence in a group of native Americans in Oklahoma, U.S., has been estimated to be above 2 000 per million.[16] Most studies from America and southern Europe however have estimated SSc prevalence to be around 200 per million and annual incidence to be at least 10 per million and year.[17] Reports from northern Europe and Great Britain have estimated disease prevalence somewhat lower, around 100 per million and estimated incidence to be below 10 per million and year.[18, 19] Because of this, a gradient of disease prevalence has been suggested to exist in Europe, somewhat analogous, but inverse, to more solid data regarding prevalence of multiple sclerosis.[20] It has been speculated if the variation in geographical distribution of SSc might be explained by environmental factors.[15, 21] This is yet to be proven.

Pathogenesis

SSc is a heterogeneous disease which can manifest itself to both the patient and the clinician in many different ways. Nevertheless, the rationale for including different subtypes of SSc under the same umbrella might be justified by our current knowledge of the pathogenesis of SSc.[9] Most manifestations of SSc exhibit signs of immune activation, vascular dysfunction and increased deposition of extracellular matrix (ECM) resulting in fibrosis (Figure 1).[22] This triad was described already decades ago.[2] Current knowledge also suggests that while inflammatory processes, together with environmental factors, may initiate the disease process by causing vascular dysfunction, fibrosis develops in later stages.[23] It is also possible that inflammation may actually be an epiphenomenon to a disease process initiated and propagated by vascular dysfunction.[24] Clinically this could be evident by the fact that Raynaud's phenomenon may precede other disease manifestations by many years, especially in lSSc. This concept is important because it could imply that the disease is most susceptible to pharmaceutical intervention in the early phases.

Still, current data suggests that this interpretation is a simplification of a complex interplay between different disease processes.[23] For example, the temporal dynamics of SSc manifestations are very different between organ systems such as

the skin, heart, gastrointestinal (GI) tract and the lungs.[25] Nevertheless, the perspective of early and late disease might give us a framework which facilitates understanding of the pathogenic processes in SSc.[9]

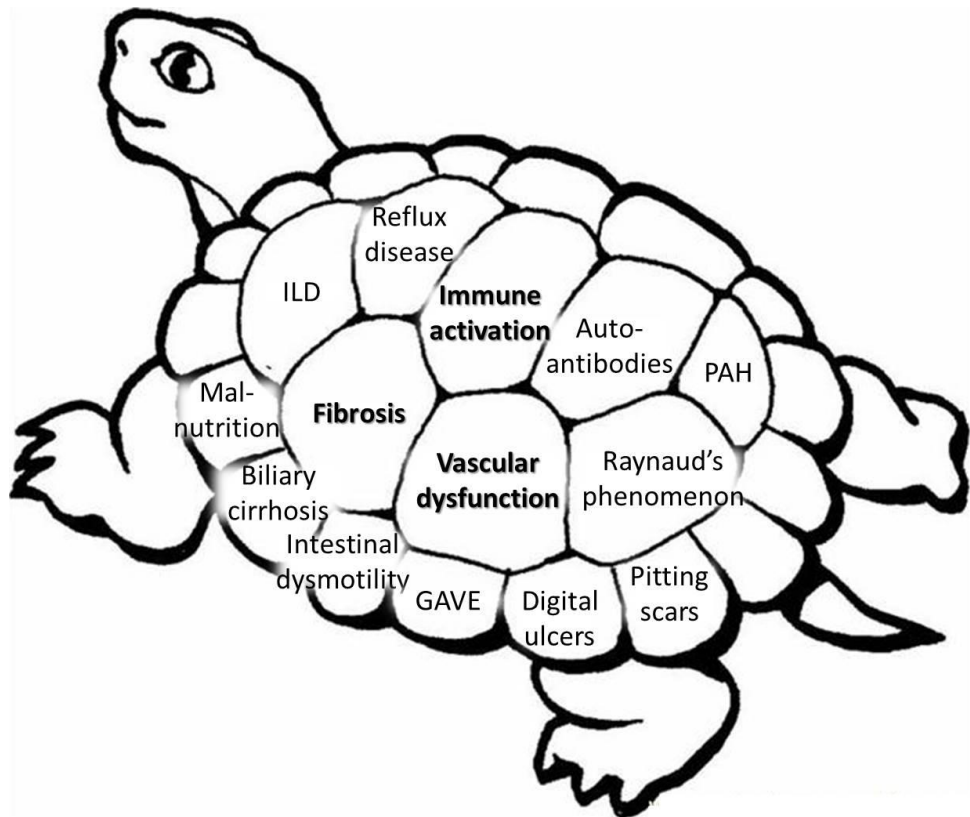


Figure 1. The burden of systemic sclerosis

Systemic sclerosis is a heterogenous disease with a complex pathology. Different combinations of vascular dysfunction, immune activation and fibrosis contributes to a multitude of various clinical manifestations. The turtle was chosen by the Swedish patient organisation as a symbol for the disease.

GAVE: Gastric antral vascular ectasia, ILD: Interstitial lung disease, PAH: Pulmonary arterial hypertension.

Early disease

Vascular injury has been shown to be a crucial, early event in SSc. The exact cause of the vascular perturbation remains to be elucidated, and is probably multifactorial. Specific environmental stress factors such as exposure to silica or vinyl chloride is known to cause SSc or SSc-like disease.[26, 27] Other proposed aetiologies include viral infections and certain drugs. Furthermore, it has been suggested that allogeneic cells, originating from the foetus of earlier pregnancies may induce SSc.[28] Although there is independent data that supports this theory,

this hypothesis remains to be proven. Nevertheless, there are several similarities between graft-versus-host disease and SSc, including skin fibrosis and GI disease.

In early SSc vascular endothelial cells undergo apoptosis which will disturb the integrity of the microvascular lining. This might cause platelet activation which may further disrupt the microvascular blood flow. Perivascular regions will show infiltration of immune cells, most notably T cells, macrophages, B cells and mast cells. Furthermore, smooth muscle cells and pericytes in the small blood vessels will proliferate, resulting in a thickened vascular wall. Proliferating pericytes may differentiate into myofibroblasts and fibroblasts, which will initiate production of ECM. This production will contribute to vascular obliteration, reduced elasticity of blood vessels and cause a paucity of small blood vessels in the affected tissue, resulting in ischaemia.[9, 29] The balance between dilating factors (such as NO) and molecules such as endothelin will also be disturbed.

Obliteration of the vascular system might lead to hypoxia. Tissue hypoxia will cause an increased production of reactive oxygen species, which will damage endothelial cells but stimulate smooth muscle cells and fibroblasts to produce ECM and, eventually, contribute to a vicious circle.[30, 31]

The production of ECM is heterogeneous in early disease and includes different types of collagen, elastic fibers and proteoglycans attracting fluid to the tissue. The clinical equivalent of this initial production might be the typical swelling of the hands in early SSc, often referred to as puffy fingers.[29]

A large number of soluble factors have been suggested to be involved in the processes outlined above. Among these, transforming growth factor beta (TGF- β), platelet-derived growth factor (PDGF) and connective tissue growth factor (CTGF) may be the most important.[32-34] There exists an interplay between the ECM and these factors. For example, extracellular TGF- β can be retained in areas where ECM is deposited, resulting in high local concentrations of TGF- β .[35] The role of PDGF is underlined by the possible presence of stimulatory antibodies to the PDGF receptor in SSc subjects, exemplifying another way by which the immune system might interact with the production of ECM.[36] Also, non-soluble factors, such as components of the ECM, can interact with and propagate the fibrotic process by outside-in and inside-out signalling generated by interactions with transmembrane receptors such as the integrins.[37]

Late disease

In later stages of SSc, deposition of collagen fibrils will dominate the remodelling of the ECM.[38] The arrangement of collagen I will be different compared to healthy skin, as increased expression of lysyl hydroxylase 2 will render the fibrotic tissue rich in cross-links and consequently less elastic and less degradable than other collagenous tissues.[39]

Perivascular ischaemia and fibrosis may eventually affect neighbouring neurons. As a consequence, the patient might suffer from a dysfunctional autonomic nerve system, which can complicate Raynaud's phenomenon, contributing to tissue ischaemia.[40] It is possible that similar processes take place in the GI tract, contributing to the inability of the enteric nervous system to act in a synchronised, physiological way, resulting in e.g. dysphagia.[41]

In skin fibrosis, the natural course of the disease usually ends with a regression of the inflammatory reaction and a subsequent deceleration of the deposition of ECM. From a clinical view-point, the end-stage of skin scleroderma is often characterised by thin, brittle, atrophic skin, tightly attached to the underlying tissues, often with secondary contractures of joints in the affected area.[42]

Immunological aspects

The exact role of the immune system in SSc is debatable. Activation of the adaptive immune system is evident by production of autoantibodies as well as the presence of B-cells and CD4+ T-cells in affected tissues. Recent data complicates this picture showing that also the innate immune system is active in SSc.[43, 44] This activation is at least partly mediated by Toll like receptors (TLR), maybe most importantly TLR2, TLR3 and TLR4.[45, 46] These receptors can be activated by exogenous stimuli, but also endogenous molecules. Such molecules are nowadays often referred to as damage associated molecular pattern proteins (DAMPs). Several of these molecules can be found in the developing ECM, most notably hyaluronan but also fibronectin.[47] Another group of DAMPs originate from immune cells. One important DAMP candidate is calprotectin, the heterodimer of S100A8 and S100A9, which binds TLR4.[48] The possible contribution of calprotectin to the pathogenesis of SSc remains to be elucidated.

Molecular analyses of skin from subjects with SSc have contributed to increasing knowledge on both a transcriptional, molecular and protein level on the pathogenic pathways involved in SSc pathogenesis. Interestingly, clinically unaffected skin shows many similarities with pathological skin in regard to gene expression.[49] It remains to be explained why, within the same patient, very similar pathological gene expression might result in quite different phenotypes in skin with different location. Correspondingly, it remains to be explained why skin fibrosis and vascular dysfunction preferably involves the hands and fingers. One explanation could be that fibroblasts situated on distinctive parts of the body have different intrinsic properties in relation to e.g. ECM production.[50]

Clinical features

SSc is a heterogeneous disease. Several internal organs as well as the skin can be subject to both vascular perturbations and fibrosis following the time course of the disease. SSc has among the highest mortality rates of the rheumatic diseases, comparable with many neoplastic disorders.[51] The most common manifestations responsible for SSc-related deaths are interstitial lung disease (ILD), scleroderma renal crisis, PAH and severe GI disease.[5]

While ILD and renal involvement often are manifested in early disease and in patients with dSSc, PAH typically develops in later stages of disease in patients with the lSSc subtype. Severe GI complications to SSc have been reported in both early and late disease, in both lSSc and dSSc.[3] The subject of this thesis mainly discusses SSc manifestations in the lung and GI tract. SSc disease in these organ systems are thus further described below.

Interstitial lung disease and SSc

Clinically significant ILD is estimated to be prevalent in 25 % of SSc patients, being more common (35 %) in dSSc than in lSSc. Autopsy reports have indicated that histological signs of ILD are present in up to 80 % of cases.[41] ILD is the most common cause of disease-related death in SSc.[52] ILD is usually bilateral and begins in the basal layers of the lungs.

Non-Specific Interstitial Pneumonia (NSIP) is the most common subtype of ILD in SSc, but patients can also be affected by Usual Interstitial Pneumonia (UIP), which is associated with a worse prognosis.[53]

Diagnosis and further assessment of ILD in SSc is preferably made with high-resolution computed tomography (HRCT) together with pulmonary function tests. Interestingly, analysis through HRCT is often able to differentiate the subtype of ILD and is helpful to select patients who may benefit from treatment.[54] Lung biopsy is thus rarely needed. Extensive disease identified on HRCT is a predictor of mortality.[54] Further characterisation of lung fibrosis can be made with bronchoalveolar lavage. Data obtained from such analyses may give important information regarding the pathogenesis of SSc, but are seldom indicated in routine clinical care.[55]

Treatment

Two of few randomised clinical trials in SSc showed a significant but small benefit of immunosuppression with cyclophosphamide compared to placebo in SSc-ILD.[56, 57] This immunosuppressant is toxic and not suitable for treatment of mild SSc-ILD. The inability to predict the natural development of mild or early manifestations of SSc-ILD makes it a most delicate question to decide which

patients with SSc-ILD should be treated. Watchful waiting and frequent assessment of pulmonary function is in many cases the preferable alternative.[54] If immunosuppression is indicated, both mycophenolat mofetil and azathioprine have been suggested as alternative immunosuppressants. In comparison to cyclophosphamide, these agents are less toxic and can thus be used for longer time periods. More than one year of immunosuppressive treatment is probably necessary in order to significantly change the natural course of SSc-ILD.[58]

Although histopathological data and experimental animal models suggested that inhibition of endothelin-1 could improve outcome in SSc-ILD, a study of the endothelin receptor antagonist bosentan failed to prove such an effect in a prospective open-label study.[59, 60] In selected cases, lung transplantation is a life-saving treatment of SSc-ILD.

In the clinical situation, the decision to initiate, continue or discontinue immunosuppressive therapy depends on several factors such as side effects and the individual response to treatment. In this situation, any tool that reflects not only existing lung damage, but also current disease activity and prognosis would be most valuable.

Gastrointestinal disease and SSc

In the vast majority of cases, SSc affects also the GI tract. For most of these patients, SSc GI disease substantially contributes to a decreased quality of life.[61, 62] GI manifestations are common in both lSSc and dSSc and relatively similar between these two subsets.[63] Although some studies indicate that fewer SSc patients die of SSc GI disease today than some decades ago, GI manifestations of SSc remain a significant direct and indirect cause of SSc-related death.[3] A correct estimation of the contribution of GI dysfunction to overall mortality is hard to make; patients with dysphagia may succumb to fatal aspiration pneumonia and patients with occult GI bleeding may suffer from anaemia.[3, 5, 52, 64] Patients with severe GI manifestations of SSc have an especially poor prognosis with only 30 % of such patients surviving more than 3 years after onset of severe GI disease.[65]

The definition of GI involvement in SSc is not unproblematic. Although oesophageal manifestations of SSc are common, normal oesophageal examination or absence of oesophageal symptoms do not rule out SSc GI disease. One possibility is that another, not so easily accessible part of the GI tract might be involved. Another possibility is that the oesophagus is affected by SSc although not causing any symptoms. Indeed, a recent study showed that pathologic oesophago-gastro-duodenoscopy was prevalent in 9 out of 13 newly diagnosed SSc subjects who all were without upper GI symptoms.[66] Also, pathologic

oesophagus motility has been observed in parts of the oesophagus where no pathology could be identified at autopsy.[67]

Although sometimes (also in this thesis) referred to as a single organ manifestation, SSc can manifest itself in many different ways in the GI tract, both with regard to pathogenesis and with regard to symptoms, often changing over time.[62] The knowledge on the pathology of SSc in the GI tract is limited compared to other organ systems. The main reasons are associated with the inherent difficulties in obtaining both relevant histological material and radiological data from this part of the body. I will present a brief overview of suggested mechanisms behind GI pathology, as well as a description of common clinical manifestations in order to facilitate critical appraisal of the work presented in this thesis.

Pathomechanisms: Vascular dysfunction

Histological studies have shown that the microcirculation throughout the GI tract can be disturbed in SSc.[68] The vascular endothelial cells appear swollen and the basement membranes in the capillaries are altered.[69, 70] The small arteries may show narrowing of the lumen. These histological findings have been corroborated by functional studies that have shown pathological GI blood flow in patients with SSc.[71, 72] Furthermore, vascular pathologies, sometimes resembling telangiectasias, can be present in the GI tract.[73-76] In the stomach, development of gastric antral vascular ectasias may occur. These are sometimes referred to as “watermelon stomach”.[77]

Immunohistochemical examinations of the ventricle wall have shown molecular similarities between SSc GI disease and PAH, as well as Raynaud’s phenomenon.[78] It has been suggested that therapies that are effective against classical vascular manifestations of SSc might be effective also against GI manifestations of SSc.[44]

Pathomechanisms: Autoantibodies, neuropathy and inflammation

Both dysmotility and absence of normal motility in the GI tract is common in SSc, not only in the oesophagus but also in lower parts of the intestine, accounting for several of the clinical situations described later.[62, 79-81] These dysrhythmic manifestations are in many cases caused by pathologic activity of the enteric nervous system, which can be assessed by e.g. electrogastrography.[82]

The production of autoantibodies is a hallmark of SSc.[83] There is some evidence that autoantibodies in SSc may cause GI dysfunction by interacting with the enteric nervous system.[84] More specifically, the muscarinic acetylcholine receptor has been proposed to be a target of autoantibodies in patients suffering from severe GI disease.[85]

One alternative, or additive, mechanism by which SSc might disrupt the enteric nervous system and thus the motility of the GI tract, is through ischaemic damage to the vasa nervorum.[41, 62] Damage to the enteric nervous system has been associated with pathological levels of GI regulatory peptides.[86]

The limited number of autopsy reports and histopathological analysis of GI tissue in SSc have consequently revealed signs of on-going inflammation in the intestinal wall.[41, 67, 69, 70, 87] Further characterisation of these cells has recently revealed an overrepresentation of CD4+ T-cells together with B-cells, mast cells and eosinophils.[88]

Pathomechanisms: Fibrosis

Similar to other organs involved in SSc, the GI tract will in many cases eventually be subject to fibrosis. A recent endoscopic study using ultrasound has shown that the intestinal wall in the upper GI tract is thicker in SSc subjects compared to healthy controls, corresponding to increased deposition of ECM. Importantly, the study confirmed increased thickness of not only deeper layers of the GI wall, but also the submucosa.[89] Histological studies have shown that fibrotic transformation is possible throughout the GI tract including the oesophagus, ventricle, duodenum, jejunum, large intestine and anal sphincter.[62, 90]

ECM deposition will inhibit the motility of the intestine, contributing to symptoms such as constipation and bloating. Fibrosis has also been suggested to affect the capability of the small intestine to absorb nutrients. In the anal sphincter, fibrosis may contribute to anal incontinence.[91]

In the GI tract, development of fibrosis is usually coupled with muscular atrophy. Mechanical studies are in agreement with these histological data, indicating changed properties of the ECM and decreased muscular function.[92, 93] The combination of fibrosis and muscular atrophy has been suggested to contribute to an increased risk of intestinal perforations in SSc.[76]

Clinical picture: The oesophagus

GI involvement of the oesophagus is a common cause for decreased quality of life in the SSc patient.[94] SSc typically affects the lower parts of the oesophagus. The patient may suffer both from dysfunctional motility and of a lower oesophageal sphincter incapable of preventing gastric acid from reaching the oesophagus. While dysmotility is more common in early disease, hypomotility or aperistalsis of the oesophagus is suggested to be more common in later stages of disease. The resulting symptom for the individual patient is often dysphagia, which can be severe and especially detrimental for SSc patients who suffers from malnutrition.[67] Furthermore, most SSc patients are prescribed several medications to be taken orally. Ingestion of oral medication may not only be

painful and time consuming for the patient. SSc patients with dysphagia are at an increased risk of having tablets stuck in the oesophagus, resulting in local damage.

Patients with SSc are at increased risk of oesophageal reflux disease. This is true also for patients who do not have any oesophageal symptoms at all.[95] The mechanisms behind this increased risk are at least partly explained by a lower tonus in the lower oesophageal sphincter together with delayed emptying of the ventricle. Reflux disease is not only painful and debilitating. Patients with reflux disease are at an increased risk of developing strictures as well as Barrett's oesophagus, a pre-malignant condition.[62, 96] It has also been suggested that reflux disease may result in acid material reaching the lungs through the oesophagus and the trachea. This might result in, or complicate already existing, lung fibrosis.[97] Severe reflux disease is also a contraindication for lung transplantation as the reflux chemically can trigger an immunologic rejection.

Dysfunction of the oesophagus has also been suggested to contribute to the increased prevalence of candida oesophagitis in SSc. The symptoms of candida infection are in some cases subtle although some patients experience dysphagia, nausea and painful swallowing.[98] Correct diagnosis can be done through endoscopic investigation but also through cineradiography.[99] Candida oesophagitis is an important example of secondary complications to SSc and highlights the fact that not all symptoms in the SSc patient represent primary manifestations of SSc. For the clinician, this discrimination is challenging but sometimes quite important to consider.

Clinical picture: Small Intestinal Bacterial Overgrowth

The large intestine harbours billions of bacteria. In contrast to the colon, the small intestine is normally not a friendly environment for bacteria. Bacteria ingested through the mouth are subject to the harsh acid environment of the ventricle before reaching the small intestine. Retrograde colonisation of bacteria from the colon into the ileum is counteracted by regular antegrade movement of the small intestine.[62]

Different aspects of bowel dysfunction in SSc may promote bacteria to enter, survive and multiply in the small intestine. These bacteria may compete with the host regarding nutrient absorption. Furthermore, the bacteria may produce large amount of gases which easily will cause pain in a SSc patient with a non-elastic intestinal wall.[93] Other common symptoms include diarrhoea, flatulence and bloating. These symptoms might be aggravated after ingestion of milk products containing lactose.

Clinical diagnosis can in many cases be confirmed by a hydrogen breath test, but there exists no consensus on an exact definition, or a gold standard, to diagnose this syndrome.[100] Bacterial overgrowth of the small intestine is also common in patients suffering from e.g. diabetes mellitus or post-surgical intestinal adhesions.

Interestingly, three independent studies have failed to demonstrate any association between small intestinal bacterial overgrowth and the faecal biomarker calprotectin.[101-103]

Clinical picture: Pseudoobstruction

Pseudoobstruction is an uncommon, but severe and potentially fatal complication of SSc.[3, 65] Several pathomechanisms are likely to contribute to this situation, including neuropathy, bacterial overgrowth and muscular atrophy of the intestine. The patient usually presents with subacute development of nausea and stomach pain. Radiological examination shows dilated bowels proximal to the point of pseudoobstruction.

The correct diagnosis of this GI complication of SSc is important but challenging. In 2012, diagnostic criteria for chronic intestinal pseudo-obstruction were presented.[104] These criteria can be summarised as

- a) a more than 6 month history of GI complaints, including
- b) bloating or pain, together with
- c) radiological signs of intestinal dilatation and/or air-fluid levels
- d) absence of structural abnormalities

Treatment is not clear-cut, but may involve total parenteral nutrition and antibiotics. Home parenteral nutrition may be a life saving therapy in the most severely affected patients. Surgery should be avoided if possible since the obstruction is not mechanical but rather a consequence of impaired movement in the intestine. Relapse is common.[105]

Clinical picture: The liver and pancreas

Patients with anti-centromere positive ISSc are at an increased risk for primary biliary cirrhosis, a combination that sometimes is referred to as Reynold's syndrome.[106] Cirrhosis may be especially critical for SSc patients since several medications are dependent on a preserved liver function.

Pancreas insufficiency is a possible manifestation of SSc. Failure of this exocrine organ may aggravate malnutrition.[107]

Clinical picture: Anal incontinence

During the last decade, a number of independent investigators have reported an overrepresentation of anal incontinence among patients with SSc.[108, 109] Recently, a questionnaire based study that included patients with other rheumatic diseases, showed that such symptoms were prevalent also in patient with other rheumatic diseases.[94] As with the oesophagus, the anal sphincter in SSc might be subject to dyscoordinated muscular actions as well as muscle atrophy and

fibrosis.[108] Furthermore, the demands of the anal sphincter are increased if the propulsive activities of the colon are pathologically intensified or weakened. The impact on quality of life from these manifestations has recently begun to be recognised as substantial and in many cases superior to other manifestations of SSc.[109]

Clinical picture: Malnutrition

Malnutrition is a common complication to SSc and is associated with increased mortality.[110] A study based on a questionnaire has estimated that 18 % of SSc subjects are at high risk for malnutrition.[111] In another study, estimations based on objective assessment of malnutrition through bio-electrical impedance measurements indicated the prevalence of malnutrition to be above 50 %.[110] These reports also indicate that malnutrition screening through assessment of BMI and S-albumin alone is insufficient.[112]

The mechanisms behind malnutrition are so far only poorly explored. A recent British study suggested that several extra-intestinal factors might contribute to malnutrition.[108] Such factors could be SSc induced hypercatabolism, depression-related disorders, decreased ability to maintain activity of daily life, and concomitant SSc manifestations such as fingertip ulcers, microstomia and fatigue.[112, 113] SSc GI disease can, at least theoretically, cause malnutrition in several ways, including dysphagia, gastroparesis, small intestinal bacterial overgrowth, dysmotility of the small and large intestine, pancreatic insufficiency and disturbed balance of gut hormones. Importantly, malnutrition is a common feature in patients with chronic intestinal pseudo-obstruction.[104] Needless to say, correct management of the individual patient is challenging. Focus on the most important underlying mechanism in each individual patient is essential. [64, 114] Access to adequate nutritional guidance should be implemented already early in disease.

Treatment of GI manifestations of SSc

As of today, we have insufficient treatment possibilities against GI SSc. Proton pump inhibitors (PPI) are important in relieving reflux symptoms and may decrease the risk of complications from reflux disease. Gastroparesis and intestinal dysmotility might be relieved by prokinetic agents. Bacterial overgrowth of the small intestine usually responds well to cyclic treatments with antibiotics.[58] Micronutrient deficiency is often helped by substitution of vitamins and/or minerals.

It should be acknowledged that several of the available treatment options for SSc GI are not pharmacological. Bleeding from the gastric ectasias is best treated by endoscopic intervention.[115] Total or partial parenteral nutrition is essential in the treatment of severe malnutrition.[105] In some cases, parenteral endoscopic gastrostomy may be life-saving.[114] In other cases, balloon dilatation of

strictures might be equally important. Both reflux disease and gastroparesis may be relieved by lifestyle recommendations such as sleeping with the head raised and avoiding food triggering reflux and large sized dinner portions.

The above mentioned recommendations are complicated by the fact that treatment with PPIs lower the pH of the stomach and can thus increase the risk of developing small intestinal bacterial overgrowth. Furthermore, frequent antibiotic treatment of bacterial overgrowth might stimulate the development of bacteria resistant to antibiotics. This can be especially detrimental in immunosuppressed SSc patients who are at an increased risk of developing serious infections e.g. in the lungs or from digital ulcers.

Finally, several medications commonly used in SSc have GI side-effects that can be hard to differ from primary manifestations of SSc GI disease. These include glucocorticoids and NSAIDs that might aggravate GI bleedings, but also the immunosuppressant mycophenolat mofetil that often is associated with secondary diarrhoea.

Monitoring disease

Monitoring patients with SSc is important in order to identify involvement of internal organs as early as possible. Correct assessment of disease activity and severity is also important in order to evaluate prescribed treatments in both the individual patient and in any clinical study. Monitoring such a heterogeneous yet uncommon disease as SSc is challenging and not without problems both for the patient and the treating physicians.

A successful follow-up of the SSc patient is dependent on several factors. I believe these include a sincere relationship between the physicians, the paramedical personnel and the patient, resulting in the possibility for the patient to report the impact of both the disease and its treatment in daily life. Another important factor is the possibility to investigate the patient in a structured yet personalised scheme. Finally, in order to monitor disease activity and severity over time, access to validated and objective investigations are crucial. For patients with chronic diseases, such as SSc, where several organ systems are to be investigated repeatedly – it is also crucial that these analyses are feasible and harmless.

Specific proteins measurable in body fluids can in many cases be analysed both objectively and feasibly. In this thesis I wanted to explore the potential of two such biomarkers, namely measurement of an extracellular matrix protein in sera and an inflammatory protein in faeces.

Biomarkers in SSc

Definitions

The term *biological marker*, often shortened to *biomarker*, has been used for many years with a certain degree of variation of what we understand and refer to with this term. Almost fifteen years ago, the NIH and FDA agreed on one definition which is most commonly used today. A biomarker is defined as:

a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention.[116, 117]

Sometimes the term *surrogate endpoint* is used. This usually refers to:

a biomarker that is intended to serve as a substitute for a clinically meaningful endpoint and is expected to predict the effect of a therapeutic intervention.[117-119]

In this context, the term *endpoint* refers to:

a clinically meaningful measure of how a patient feels, functions, or survives.[117-119]

Sometimes the term *soluble biomarker* is used to discern biomarkers measurable in bodily fluids from radiological findings and physiological investigations.[119, 120]

In modern healthcare, several biomarkers are already in use. Important examples are C-reactive protein – a biomarker for inflammation, and urate – a biomarker for chronic gout.[121] A good biomarker can serve as a diagnostic tool but also give the clinician valid information regarding disease activity and prognosis. Such information is crucial when to select treatment strategies for the individual patient. Finally, a good biomarker can be helpful when evaluating initiated drug therapy.[122]

Besides this clinical situation, feasible biomarkers are also useful in the field of drug development as a biomarker can simplify the process of evaluating novel drug candidates, both in experimental models and in clinical trials.

In order for a biomarker to be useful, the reliability, validity and responsiveness to change must be established.[123] An “informal gathering of professionals interested in outcome measures in rheumatology”, collectively referred to as OMERACT (Outcome Measures in Rheumatoid Arthritis Clinical Trials), have suggested and defined key variables to be used in this evaluation process in the field of rheumatology. Their approach, referred to as the OMERACT filter,

focuses on three elements, namely *truth*, *discrimination* and *feasibility*. [124] This approach has also been discussed in reference to SSc. [125]

Their term *truth* refers to whether the biomarker measures what was intended and if it is unbiased. This issue deals with the concept of validity. Validity can further be explored by differentiating between face validity, content validity, construct validity and divergent validity. [122, 126] Briefly, face validity deals with the concept of whether the properties bestowed on the biomarker “make sense”. Content validity can refer to what degree the biomarker is applicable to different clinical situations for a given disease. Construct validity may discuss how well a biomarker correlates with a gold standard. Finally, divergent validity may refer to the capability of a biomarker to correctly differentiate between two different groups of patients. [126]

The term *discrimination* can refer to the capacity of the biomarker to identify patients with low and high risk for complications, as well as to recognise a change in disease activity in the individual patient. In order to discriminate between different situations, it is necessary that the biomarker has a high degree of reproducibility, e.g. a sample analysed at different laboratories should give the same result. [124]

The term *feasibility* can refer to how easy the biomarker can be applied in clinical practice, and refers also to costs and how easy the results are to interpret. [126]

The development and validation of feasible biomarkers in rheumatology in general has proven more complex than initially expected. For SSc, this challenge is even greater considering the multitude of organ manifestations, the heterogeneity and rareness of the disease as well as the limited knowledge on the pathogenesis behind the many SSc manifestations. Some organ systems have been extensively discussed in reference to an unmet need of feasible biomarkers. [125] I want to discuss one of these organ systems further.

Biomarkers for skin fibrosis

The modified Rodnan Skin Score (mRSS, (described in later chapters)) has received universal recognition as the gold standard for the assessment of skin fibrosis in SSc. As this measurement tool has some inherent weaknesses, several attempts have been made to monitor skin fibrosis in SSc through soluble biomarkers. [127]

One group of molecules measurable in serum are cytokines and growth factors. Serum levels of important mediators in SSc, such as TGF- β and CTGF, have shown some association with skin fibrosis. The results have however not been self-evident to interpret. One complicating factor is that the level of a cytokine in serum might be very different from the local concentration in affected skin.

Another complicating factor is that a substantial part of both TGF- β and CTGF are fragmented in the sera, which might explain why some studies using different antibodies in the analysis of these molecules have shown conflicting results.[128-130] Other cytokines that have shown promising potential as biomarkers in SSc skin disease are interleukin-6, interleukin-10, interleukin-13 and B cell activation factor.[131-133]

An alternative approach to inflammatory mediators is to investigate molecules involved in the organisation of the extracellular matrix. One large group of such potential markers are the collagen metabolites. During the production of collagen in the skin, N- and C-terminal propeptides, are cleaved from the procollagen and released into the circulation. The N-terminal propeptide of collagen type III has repeatedly been shown to correlate with skin fibrosis [43, 134]. After cleavage of the propeptides, the triple helical domain of collagen is flanked by telopeptides. Serum concentrations of both the isolated and the crosslinked C-terminal telopeptide of collagen type I have been shown to reflect skin fibrosis to a certain degree.[135, 136]

Metabolism of extracellular matrix is regulated by several enzymes and inhibitors of these enzymes. One such inhibitor, tissue inhibitor of metalloproteinases 2 is upregulated in the skin of patients with SSc. Increased serum levels of this inhibitor has been identified in SSc, and levels seem to correlate with extent of skin fibrosis.[137] Similarly, matrix metalloproteinase 9 has been shown to be increased in SSc serum and to correlate with skin sclerosis.[138]

Cartilage oligomeric matrix protein (COMP) is a pentamer that is involved in the assembly of collagen fibrils. Serum-COMP (S-COMP) has been shown to have a superior correlation with skin fibrosis compared to the aforementioned molecules, and will be discussed further in the next chapter. [139]

Finally, the production of ECM in skin and other organs does not only involve proteins but also proteoglycans. Xylosyl transferase is an enzyme that is involved in the attachments of sugar chains to proteins, forming proteoglycans.[140] Increased activity of this enzyme has been demonstrated in serum of SSc-patients. Correspondingly, increased serum levels of hyaluronan have been recognised in SSc almost 30 years ago.[141]

An alternative approach in the search for feasible and valid biomarkers of skin fibrosis has been to investigate available biomarkers in other diseases and to combine different biomarkers. The Enhanced Liver Fibrosis (ELF) test measures serum levels of procollagen III N-terminal propeptide, tissue inhibitor of metalloproteinase 1 and hyaluronan. It is a validated non-invasive serum test used in the assessment of liver fibrosis. A recent report suggests that this test could also be valuable in SSc.[142] The correlation between ELF and skin fibrosis was substantially lower than the correlation between COMP and skin fibrosis ($r=0.28$ vs $r=0.60$ respectively).[139]

Another alternative in the evaluation of skin fibrosis is to analyse skin biopsies. A recent report from Boston has shown promising result when measuring mRNA-levels from a combination of four different proteins in skin biopsies.[143]

Finally, several attempts have been made in order to directly measure skin thickness objectively by different tools such as ultrasound and durometer.[144] It can be speculated that such methods could function synergistically with soluble biomarkers.

Cartilage oligomeric matrix protein (COMP)

Cartilage oligomeric matrix protein (COMP) is a molecule consisting of five identical subunits that are attached to each other in the N-terminal through a coil-coil domain that is further reinforced by disulphide bridges. In the C-terminal ends, COMP has five globular domains that can interact with components of the extracellular matrix. The structure of COMP can be compared with that of a bouquet of five tulips, intertwined in the base and with five sticky, adhesive flowers in the other end (Figure 2).[145]

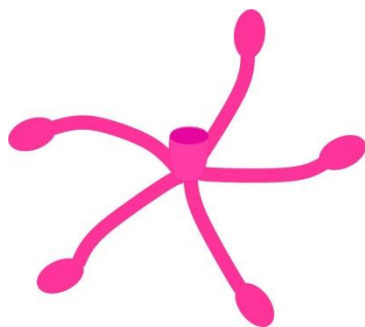


Figure 2. Cartilage oligomeric matrix protein

COMP is a pentamer consisting of five chains attached to each other in the N-terminal. Please note that each monomer can bind a collagen molecule, but also how two monomers cannot bind the same collagen molecule.[146] Schematic picture provided by Pilar Lorenzo, Sections of Rheumatology and Molecular Skeletal Biology, Lund University.

COMP was first identified in cartilage.[147] Later studies have revealed that COMP is present also in tendons as well as in the liver, the lung and the skin when these tissues are subject to fibrosis.[148, 149]

The C-terminal of COMP can bind to both collagen type I and III (that are present in skin) and collagen type II (that is present in cartilage). Importantly, the binding sites on collagen to which COMP binds are dispersed in such a way that two globular domains on the COMP molecule cannot bind to the same collagen molecule at the same time. COMP can, however, bind up to five different collagen molecules simultaneously. By binding multiple collagen molecules, COMP brings individual collagen fibrils closer to each other and catalyses the process of collagen fibril formation.[146]

While COMP can bind to individual collagen molecules it cannot bind to collagen molecules that have formed collagen fibrils. This suggests that COMP has different functions in the developing and in the mature ECM. COMP does however bind to a large number of proteoglycans in the ECM, including matrilin, the non-fibrillated collagens IX, XII and XIV, fibronectin and chondroadherin, which all can interact with the collagen fibres.[150-153] It is likely that in the mature ECM, COMP – with its long, flexible arms with sticky ends – contributes to structural stability of the collagen network.[154]

COMP has been shown to interact with the complement system, activating the alternative pathway while at the same time inhibiting the classical pathway.[155] Structural analyses of the COMP molecule have shown that the N-terminal stalk region of COMP is able to harbour both vitamin A and vitamin D₃. [156] A possible role of COMP as storage or transporter of these vitamins remains to be explored. Presumably, the ways in which COMP contributes to both health and pathology are multiple.

COMP belongs to a group of proteins known as the thrombospondins (TSP), and has also been referred to as thrombospondin 5. Both TSP-3 and TSP-4 can be assembled in a pentameric way such as COMP, while TSP-1 and TSP-2 are assembled in three-stranded oligomers. TSPs 1-4 are not as extensively studied as COMP, and possible overlapping functions may exist, although these proteins are found in different tissues.[157] TSP-1 has been studied in reference to wound healing and systemic sclerosis and has been suggested to be directly involved in the development of skin fibrosis, one mechanism being binding of TGF- β in the ECM.[158]

Mice deficient in COMP are healthy and show normal development of bone and cartilage, supporting the concept that there are redundant mechanisms in the assembly of collagens.[159]

COMP in the field of rheumatology

COMP in body fluids is measurable with enzyme-linked immunosorbent assay (ELISA).[160] Levels of COMP in synovial fluid from patients with both osteoarthritis (OA) and rheumatoid arthritis (RA) are increased. These patients do also have increased levels of COMP in serum. S-COMP has been shown to correlate with disease activity and predict radiological damage in RA.[161, 162] In OA, multiple studies have confirmed that S-COMP reflects OA development.[163, 164] Furthermore, S-COMP shows good reproducibility upon repeated measurement as it exhibits only limited diurnal variation and is quite stable even after physical exercise.[165, 166] Several ELISAs are available for the measurement of COMP, and COMP-levels are sometimes referred to in $\mu\text{g/ml}$ and sometimes U/l. This is a complicating factor when comparing different reports on

this biomarker. It reflects the fact that different antibodies are used when measuring COMP. It remains to be elucidated to what degree different ELISAs measure the intact pentameric COMP, and if larger or smaller fragments can interfere with the different ELISAs.

COMP and systemic sclerosis

COMP was identified in equine tendon already in 1997, and was later shown to be associated with scar formation in tendon.[149, 167] Eventually, COMP was identified in a gene expression analysis of human fibroblasts exposed to TGF- β . [33] Later, COMP was found in skin from SSc subjects, and also in fibrotic lung and kidney tissues from patients with SSc.[148, 168] Other studies revealed that COMP was also expressed in, and could accelerate, liver fibrosis in an experimental rat model.[169]

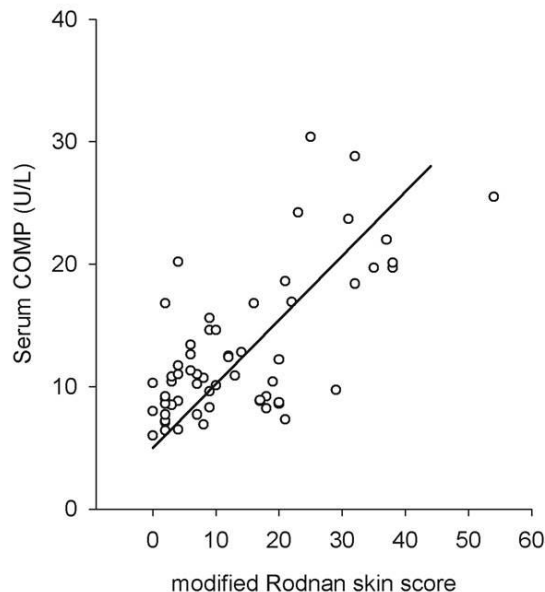


Figure 3. S-COMP correlates with the modified Rodnan Skin Score

S-COMP has shown significant and clinically relevant correlations with the modified Rodnan skin score in both longitudinal and cross-sectional studies (shown here). Adapted from Hesselstrand et. al. 2008.[139]

Serum levels of COMP were later shown to be increased especially in early dSSc, reaching values higher than levels typical of RA and OA. Furthermore, a cross-sectional and longitudinal study could show that S-COMP correlated well with extent of skin fibrosis (Figure 3).[139] These results were supported by independent data showing a clear correlation between skin fibrosis and mRNA

expression of COMP in skin biopsies. Notably however, this study also showed increased COMP expression in clinically healthy skin in SSc subjects.[168] Later studies have confirmed the presence of COMP in the deeper, reticular dermis in SSc and also in localised scleroderma.[170] In contrast to earlier reports, these studies have also detected COMP in the more superficial, papillary dermis, where it is present also in healthy subjects.[153, 170]

Calprotectin

Calprotectin was originally identified in 1980 by Fagerhol et. al. as a marker of leucocyte turnover. It was initially named L1 protein.[171, 172] The name calprotectin was proposed ten years later when calcium-binding and antimicrobial properties of the protein were described.[173] Further on, calprotectin was shown to be a heterodimer of two S100-proteins, and is thus often referred to as S100A8/A9.[174, 175] The rationale behind the name calprotectin has later been questioned – today both the terms S100A8/A9 and calprotectin are widely used.[175] In addition, the heterodimer has also been referred to as MRP8/MRP14, calgranulin A/B, CP-10, p8/p14 and cystic fibrosis antigen.

The S100-group of proteins has approximately 25 members, of which 16 are S100A-protein. The S100-proteins are small proteins of 10-12 kDa size and show at least fair homology with each other. They are all soluble in a saturated ammonium sulphate solution, hence their name. Their distribution in different tissues is variable – while S100A8 and S100A9 are expressed mainly in myeloid cells, e.g. S100A1 is highly expressed in muscular tissues and in the brain. Several mechanisms by which the different S100 proteins interact in intra- and extracellular processes in both health and disease have been identified. Common to most of the S100 proteins is the ability to bind calcium and thus undergo conformational changes which can modulate the biological properties of the protein. Furthermore, the S100 proteins can form homo- and heterodimers as well as oligomers with each other. It has been hypothesised that the amount of free monomers can be controlled this way.[176-178]

S100A8 and S100A9 are constitutively expressed in neutrophils, monocytes and dendritic cells. Lymphocytes, basophils and eosinophils do not express detectable levels of S100A8 and S100A9.[179] In neutrophils, the intracellular content of calprotectin has been proposed to be especially high, one report suggested 40-60 % of the protein mass in the cytosol to be calprotectin.[180] Intracellular calprotectin has been shown to interact with the formation of microtubuli network and intermediate filaments. These interactions could explain the decreased motility and transendothelial migration of S100A9 deficient granulocytes.[181, 182]

Calprotectin can be released from leukocytes both following activation and necrosis. Extracellular calprotectin is as a chemotactic agent for leukocytes, promoting cell migration into inflamed tissues. Maybe most importantly, calprotectin binds TLR4, and the receptor for advanced glycation end products (RAGE). In this way, calprotectin has been shown to stimulate the innate immune system and has been categorised as DAMP.[48]

The net effect of calprotectin induced TLR4 stimulation and other functions of calprotectin can be hard to predict. In a model of *E. coli* induced abdominal sepsis, mice lacking S100A9 showed significantly better survival than normal mice.[48] In a model of *Klebsiella* induced pneumonia and sepsis however, the S100A9 deficient mice showed increased mortality rates. [183] Finally, in a model of *E. coli* induced urinary tract infection, no significant differences in host response could be identified between normal and S100A9 deficient mice, even though calprotectin was induced in the urinary tract following infection in the normal mice.[184] One possible explanation could be that calprotectin exerts multiple actions, of which some actually are anti-inflammatory.[178] Another explanation could be that inflammation, depending on the context, sometimes is life saving and sometimes detrimental to the organism.

In the skin, calprotectin has been identified in inflammatory situations such as psoriasis and wound healing. Interestingly, the source of calprotectin in skin seems to be not only recruited inflammatory cells but also keratinocytes located in the epidermis. When added exogenously to the skin, calprotectin stimulates proliferation of both fibroblasts and keratinocytes.[185] Furthermore, in non-healing wounds, increased expression of S100A9 can be seen in deeper layers of the dermis.[186]

Mice deficient in fibroblast growth factor receptors 1 and 2 fail to uphold an intact epidermal barrier. These mice eventually develop skin inflammation and fibrosis. Increased expression of both S100A8 and S100A9 in the keratinocytes of these cells have been identified and postulated to be a mechanism by which these mice develop skin fibrosis.[187]

In an animal model of colonic inflammation, S100A9 was recently shown to be expressed also in colonic epithelial cells. In that model, S100A9 produced by epithelial cells following IL-6 stimulation was suggested to be an essential part of a vicious circle, recruiting leukocytes to the inflamed epithelium and activating the innate immune system.[188] Both S100A8 and S100A9 can be expressed in the intestinal epithelium in children with IBD indicating that FC levels are not only of leukocyte origin in this disease.[189]

Finally, fibroblasts stimulated by a wound healing process in rat, have been shown to express S100A8. The role of S100A8 in this process is not yet known – levels of S100A8 seem to correlate inversely with TGF- β . [190]

Calprotectin in the field of rheumatology

Increased levels of calprotectin in plasma or serum have been described in several rheumatic diseases. In systemic lupus erythematosus, S-calprotectin has been proposed to be a biomarker of disease activity.[191, 192]

Also in rheumatoid arthritis (RA), serum levels of calprotectin have been proposed to reflect and predict disease severity.[193, 194] Increased expression of calprotectin in the synovial membrane has been identified.[195] Similar findings have also been made in OA. In both these diseases calprotectin has been suggested to be an essential mediator of disease activity.[196] This concept is supported by data from an animal model of arthritis where S100A9 deficient mice were less prone to develop severe arthritis.[197]

In ankylosing spondylitis, serum levels of calprotectin have been described to be within normal range. Interestingly however, faecal levels of calprotectin were pathologically increased in a majority of these patients.[198]

Faecal calprotectin in clinical practice

Calprotectin is readily measurable in faeces. The method is simplified by the fact that calprotectin is surprisingly stable, also in faeces.[199] Even when left in room temperature for several days, the major part of calprotectin in a faecal sample remains undegraded. Furthermore the level of calprotectin in a single, 5 g sample, correlates very well with levels calculated from a 24 hour stool collection in both healthy subjects and patients with IBD.[200]

Leukocyte influx in the bowel can be estimated by labelling leukocytes with radioactive indium. Calprotectin levels in faeces have been shown to closely correlate with leukocyte influx in the bowel, and faecal calprotectin (FC) has thus been suggested to reflect inflammatory activity in IBD.[201]

Numerous studies have later showed that FC correlates with disease activity in IBD. Further studies have suggested that FC can predict disease severity, reflect response to therapy and identify relapse in IBD.[202] In an unselected population of subjects with unspecific bowel symptoms, FC has been shown to identify patients with and without organic bowel disease with high sensitivity and specificity.[203] Today, FC is routinely used in both primary healthcare and in gastroenterology. As of 2012, at least 50 000 analyses of FC were made in Sweden.[204]

Aims of the present investigation

The aims of this thesis are threefold.

- Because incidence and prevalence of SSc in Sweden have never been investigated before, and because conflicting data have been reported from different regions world-wide, we wanted to examine the epidemiology of SSc in Sweden using novel classification criteria.
- COMP has shown potential as a biomarker in SSc. We wanted to examine this potential further by:
 - 1) Exploring a possible correlation between lung fibrosis and S-COMP in SSc.
 - 2) Investigating a possible predictive potential of S-COMP in SSc.
 - 3) Investigating the development of experimental skin fibrosis in mice lacking a functional COMP gene.
- Faecal calprotectin (FC) is a feasible and reliable biomarker in IBD. Since recent reports have verified an inflammatory component in the GI tract of patients with established SSc,[78, 88] we wanted to investigate the biomarker potential of FC in SSc by:
 - 1) Exploring associations between FC and other markers of GI disease.
 - 2) Investigating the reliability and specificity of FC in SSc.
 - 3) Investigating the development of experimental skin fibrosis in mice deficient in S100A9, which lack functional calprotectin.

Methods

In this chapter I would like to discuss some aspects of the different methods and material that have been used in this thesis.

Healthcare in the Skåne region

The Skåne region is the most southern part in Sweden. As of 2012, it comprises 1.3 million inhabitants of which 1.0 million are above 18 years of age. Healthcare is mainly provided by public, and to a certain degree, private actors at a standardised low co-pay through a tax-based financing system. In the Skåne region, access to healthcare in general, and rheumatological healthcare in particular, is good.[205, 206]

The Swedish population register and the Skåne Healthcare Register

All Swedish residents are registered with a mandatory 10-digit personal identification number. The Swedish population register includes information on sex, date of birth, residential address and is continuously updated by the Swedish Tax Agency. The Swedish population register is probably the oldest continuous population register world-wide. Already in 1686 it became mandatory by law to register basically the same information as stated above.[207] The register is updated by the Swedish Tax Agency and is used by every healthcare provider as well as by authorities and companies such as the Swedish Social Insurance Agency and banks.

Since 1998, all public and private healthcare provided in the Skåne region is registered in the Skåne Healthcare Register together with the patients unique personal identification number, date of visit or discharge from hospital and name of healthcare provider. Registration is mandatory for reimbursement purposes. For public healthcare, the register also includes the patients' confirmed and tentative diagnoses according to the International Classification of Diseases (ICD) 10.

Clinical evaluation of patients with (suspected) SSc

Proper clinical examination remains a cornerstone in the diagnosis, classification and evaluation of the SSc patient. Although SSc is a disease that sometimes can be recognised on the exterior of the patient, this is not always the case. Patients with severe internal organ engagements may exhibit only limited signs of systemic autoimmune disease visible for the examining clinician. Accessory methods such as laboratory tests or radiographic analysis can sometimes be crucial in the correct assessment of SSc.

Almost all patients studied in this thesis have been subject to a strict clinical routine developed at the Rheumatology clinic in Lund. This routine includes, but is not limited to, the following procedures:

Assessment of skin thickness according to Rodnan

Thickening of the skin, scleroderma, is a main hallmark of SSc. During the course of the disease, skin thickness and quality change. Evaluation of skin thickness is considered to be an important variable in monitoring total disease activity and progression.

Assessment of skin thickness through manual palpation has been a routine procedure in both research and in the clinic. In 1979, Rodnan introduced a semi-quantitative scoring system for the complete assessment of skin thickness in SSc.[208] This scoring system has later been modified to the mRSS, and proven to be a feasible, reliable and valid outcome measure for the assessment of SSc skin disease.[209, 210] Skin involvement in SSc, as assessed by the mRSS, correlates with internal organ involvement and predicts mortality.[211]

Although the mRSS has been validated, it has some drawbacks. The mRSS measures skin thickness and not skin quality. It may not be able to discern early, oedematous SSc skin changes from later pathologies when the skin is tight, but not necessarily thickened. Furthermore, in the setting of a research trial, the mRSS might be subject to bias as the examiner, and/or the patient, may be aware, or have an opinion, regarding the effect of a recently initiated medical treatment. Finally, in recent years, clinical trials with tyrosine kinase inhibitors have highlighted the fact that adverse effects of modern medication – in this case subcutaneous swelling – can complicate the interpretation of the mRSS.[212] There is an unmet need for a feasible, reliable and valid surrogate marker for skin fibrosis that is also objective and easily repeatable.[213]

Nailfold capillaroscopy

Nailfold capillaroscopy is a non-invasive method to examine the vascular system with a more than 100 year old history.[214, 215] Although modern, computer based versions of the investigation are available, the essence is quite simple. In opposite to the rest of our bodies, in the nailfolds, our capillaries are oriented in a 2-dimensional manner. Because of this, capillary structure and configuration are accessible to examination with the help of a magnifying glass and immersion oil.[215, 216] During the last decades, it has become evident that the majority of SSc patients have pathologic capillaries as assessed by nailfold capillaroscopy. This insight has gained increased recognition worldwide.[217] “Abnormal nailfold capillary pattern” is one of the new items in the 2013 classification criteria for SSc.[11] Examination of nailfold pattern by capillaroscopy has been a routine procedure in the evaluation of SSc and suspected SSc in Lund since the early 1990’s (Figure 4).

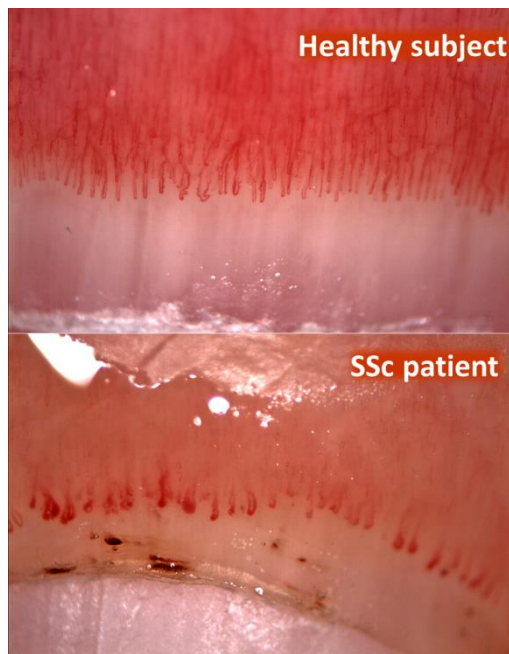


Figure 4. Normal and pathological nailfold capillaroscopy

Nailfold capillaroscopy from a healthy subject and a patient with SSc. Please notice the enlarged capillaries and focal loss of capillaries in the nailfold of the SSc patient. Photos provided by Marie Wildt, Department of Rheumatology, Skåne University Hospital, Lund.

High Resolution Computed Tomography (HRCT)

Non-invasive assessment of lung fibrosis has traditionally been done by chest x-ray. During the last decades, imaging of the lungs with HRCT has emerged and developed. HRCT based on 1 mm slices is currently the method of choice to analyse subtle changes in diffuse ILD e.g. fibrosis.[218] HRCT has increased the possibility to adequately diagnose and monitor SSc-related lung fibrosis. A semi quantitative scoring method has been developed and validated in order to analyse lung involvement in a structured way comparable between different centres.[54] In contrast to the ARA criteria from 1980, the 2013 criteria set for SSc includes assessment of ILD by HRCT.[11] At the SSc centre in Lund, lung examination by HRCT has been a routine procedure in the evaluation of SSc and suspected SSc for several years (Figure 5).



Figure 5. High resolution computed tomography

Cross sectional radiograph of the basal parts of the lungs in a SSc patient. Usage of high resolution computed tomography makes it possible to identify subtle changes indicative of incipient pulmonary fibrosis, not identifiable through conventional x-ray. Arrow points towards several traction bronchiolectasis in the lower left lung. Picture provided by Gracijela Božović, Department of Radiology, Skåne University Hospital, Lund.

Cineradiography

Cineradiography refers to a dynamic x-ray examination in which the patient swallows a radiopaque substrate, usually barium. The radiological act of swallowing is recorded with a video camera. Assessment of oesophagus motility can be studied and certain SSc-specific pathologies, such as candida and strictures, identified (Figure 6).[80, 99] Cineradiography is an objective investigation included among what has been proposed to be a “core set of variables” when to assess SSc GI disease.[219] Cineradiography only gives the examiner information about the very upper GI tract. Disease manifestations from different parts of the GI

tract have been shown to co-variate, implying that GI disease assessed through cineradiography may have some generalisability to the complete GI tract.[66, 68, 108]

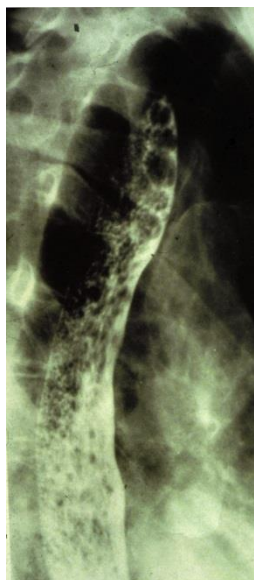


Figure 6. Barium radiography of the oesophagus

Radiological examination of the oesophagus with barium contrast in a patient with SSc. Please notice the dilated oesophagus as well as the barium staining of the mucosa, indicating candida infection.[99] Picture provided by the Department of Radiology, Skåne University Hospital, Lund.

Patient reported outcomes

In the field of rheumatology, several questionnaires have been developed, translated and validated. For SSc, the Scleroderma Health Assessment Questionnaire has gained widespread acceptance.[220] These patient reported outcomes are often intended both to support clinical decision making and to serve as outcome measures in clinical trials.

Also in the field of gastroenterology, several questionnaires have been developed for the assessment of GI symptoms. The Gastrointestinal Symptom Rating Scale has been validated in Swedish.[221]

During the last years, GI questionnaires specifically aimed for the SSc patient have been constructed and thoroughly validated.[222] So far, there is only limited data describing to what degree these subjective reports reflect objective pathologies.[223] An elegant investigation revealing the histopathological characteristics of the gastric mucosa in SSc failed to show any correlation between histologic changes and patient reported symptoms.[88] Regarding the clinical syndrome of malnutrition, the correlation between patient reported measures and physiological measurements are rather poor.[111] Based on their investigation of malnutrition in SSc, Krause and co-authors have shown that the patients' history of GI drug usage correlates with GI disease.[110] Based on these data they suggest that:

regular usage of these treatments [prokinetic drugs and PPIs in low and high doses] is probably the best marker for assessing the severity of GI symptoms.[110]

In our analyses of FC and SSc GI disease, we have used both the Swedish questionnaire and the method proposed by Krause et. al. in our assessment of severity of SSc GI disease.

Laboratory evaluation of patients with SSc

S-COMP

COMP can be measured in both joint fluids and sera by ELISA.[160] The COMP-analyses made in this thesis were made with a commercially available sandwich ELISA (Anamar, Lund) in doublets. This ELISA uses two monoclonal antibodies directed against separate antigenic determinants. From 2006 and onward, S-COMP was measured as part of the routine examination at the rheumatology clinic in Lund. All samples retrieved before this year were stored in -80° freezers and later analysed consecutively.

F-calprotectin

Patients submitted to in-hospital care at the university clinic in Lund were invited to deliver a stool sample for measurement of FC. Samples were taken according to the local instructions from the immunology laboratory and analysed with a commercial sandwich ELISA (Bühlmann laboratories, Switzerland). An important aspect in the analysis of stool samples is the necessity of pre-treating the samples in an extraction buffer followed by centrifugation in order to extract soluble calprotectin while removing non-soluble debris.[224]

Different ELISAs are commercially available for the assessment of FC. While the ELISA used in our studies uses a monoclonal calprotectin antibody, other ELISAs use polyclonal antibodies. Furthermore, the extraction buffers used are likely to contain different solutions since the exact composition is a company secret (Bühlmann laboratories, personal communication). Recent observations have revealed that different ELISA kits are not comparable, especially when used by different laboratories.[224] Preliminary data has indicated that the ELISA used in this study is superior to other ELISAs.[225]

Micronutrient deficiency

The SSc patients included in this thesis were all screened for micronutrient deficiency in regard to the following vitamins and minerals: iron, vitamin B₁₂, folate, zinc, and magnesium. Investigations were made using blood analyses and cut-off levels recommended by the local laboratory were used.

Immunological analyses

Antinuclear antibodies (ANAs) can be detected in at least 90 % of SSc subjects.[83] Identification and characterisation of these antibodies are important from a clinical point of view because different antibodies are associated with different manifestations of disease. Furthermore, in the 2013 classification criteria for SSc, reactivity towards any of three mutually exclusive autoantigens (anti-centromere, anti-scl-70 and anti-RNA polymerase III) is specified as one criterion. The patients included in this study have all been tested for the presence of antinuclear antibodies. This analysis has been made using indirect immunofluorescence using HEp-2 cells. A positive result was further characterised by the specific pattern of which homogenous, nucleolar, speckled and centromeric are the most commonly recognised. Of these, the centromeric pattern on indirect immunofluorescence is included in the 2013 criteria. Furthermore, most patients have been tested for extractable nuclear antigens – including anti-scl-70-antibodies - through immunodiffusion. Finally, a majority of the patients have been tested for antibodies against RNA-polymerase III by ELISA or immunoprecipitation.

Experimental studies

The bleomycin mouse model of skin fibrosis

Several experimental animal models of skin fibrosis have been presented. It can be questioned to what degree these models mimic SSc.[226] The bleomycin (BLM) mouse model is a well-established model of skin inflammation and fibrosis. In this model, mice develop localised skin fibrosis and possibly also autoimmunity. So far, however, no signs of vascular dysfunction have been identified in this model.[227]

BLM is a cytotoxic agent that originally was used in the field of oncology. Lung fibrosis is a relatively common side effect in patients treated with this drug. Based on these observations, intra-tracheal injection of BLM in rodents was established already in the 1980's as a mouse model of lung fibrosis. BLM was shown to induce skin fibrosis in mice already in 1973, and s.c. injections of BLM in rats was presented as an animal model of SSc in 1983.[228, 229] It was first in 1999 in however, that repeated s.c. injections of BLM in mice was presented as an animal model for localised skin fibrosis in mice. The skin fibrosis could be confirmed by histological analysis, by measurement of mRNA for collagens and by biochemical measurement of collagen content in skin.[227, 230] Further analyses using immunohistochemistry and genetically modified mice revealed that the fibrosis was preceded by a mononuclear cellular infiltrate, but also that the fibrosis could

develop in the absence of mast cells and T-cells.[231] The fibrosis was shown to be associated with transformation of fibroblasts into myofibroblasts, a process that is similar to the situation in SSc.[232] Stimulation of human dermal fibroblasts with BLM showed an upregulation of collagen I, fibronectin and decorin.[233]

Over the last decade, numerous studies have been made using the BLM mouse model of skin fibrosis. Some agents showing promise in this mouse model have now been used in clinical trials. As several mouse models are available today, it is reasonable to argue that results from more than one mouse model should be available before results from experimental models are transferred into clinical decision making in human SSc. Nevertheless the BLM mouse model remains a basic, feasible and solid model for initial, explorative studies of skin fibrosis.[226]

Assessment of skin thickness in mice

Measurement of skin thickness in mice by routine haematoxylin and eosin staining has been described in detail.[234] Briefly, from the sacrificed mouse, the sclerotic skin is removed and embedded in paraffin. From this tissue, sections parallel with the length axis and perpendicular to the skin, are carefully dissected by a microtome. Haematoxylin and eosin staining reveal skin fibrosis with dense collagen bundles, fat atrophy and sometimes epidermal hypertrophy. Measurements made by microscopy are thought to represent skin thickness in vivo. Interestingly, ultrasonographic data has recently been presented that might question this interpretation.[235] Nevertheless, histological measurements remain the gold standard for measurement of skin thickness in this model.[230]

Assessment of skin collagen content in mice

Unlike most other proteins, the collagens are rich in hydroxylated proline. Estimation of collagen content in skin can thus be estimated by measurement of the hydroxyproline content in the tissue. Sensitive measurement of this imino acid was introduced in the 1950's and later modified slightly. The method is both robust and accurate and can detect also small amounts of the imino acid. In order to measure total hydroxyproline content, tissue needs to undergo complete hydrolysis in e.g. HCl overnight. In contrast to other amino acids, after oxidation, hydroxyproline will form a chromophore when reacting with *p*-dimethylaminobenzaldehyde. The colour of the solution will thus reflect the hydroxyproline concentration and can be readily measured with a spectrophotometer.[236] Collagen content can be estimated based on the assumption that the collagen mixture in mouse skin contains 12.5 % hydroxyproline, and expressed in relation to wet or freeze-dried weight of the sample.[237]

Evaluation of inflammatory cells in mice

Since the BLM model of skin fibrosis induces not only fibrosis, but also inflammation, we wanted to investigate the dynamics and composition of the cellular infiltrates in the skin. For this purpose, we used immunohistochemistry. In principle, sections from paraffin-embedded tissues were deparaffinised and subject to antigen retrieval in a citrate-buffer for 4 hours at 60° C. Sections were blocked with 1 % BSA and 5 % serum from appropriate species. Then sections were incubated overnight at 4° C with a specific primary antibody. Next, sections were washed and endogenous peroxidase activity quenched by treatment with 0.3 % H₂O₂. Slides were then incubated with the appropriate secondary antibody, coupled to horse radish peroxidase. Finally, sections were treated with 3,3'-diaminobenzidine to visualise the primary antibody. Slides were scanned with a digital microscope and randomly analysed for the number of positively stained cells.

The following cells were analysed with commercially available antibodies – T-cells (anti-CD3-antibody), macrophages (anti-F4/80-antibody) and myofibroblasts (anti-smooth-muscle-actin antibody). Furthermore, sections were analysed for the presence or absence of S100A8 and S100A9 with polyclonal in-house rabbit antibodies (kind gift from Prof. Dr. Johannes Roth and Dr. Thomas Vogl, Münster, Germany).

Statistics

In order to draw any conclusions from the data obtained in this thesis, correct usage of appropriate statistical methods is necessary. The following aspects have been of importance when choosing statistical methods.

Descriptive analyses

Descriptive analyses can refer to the intention to describe a large set of numerical data, or observations, from a larger population, e.g. a group of patients, in a short form. Depending on the distribution and the variation of the variable studied, such data can preferably be described either by using the terms median and quartiles (non-parametric statistics) or mean and standard deviation (parametric statistics). Analysis of the data in reference to its distribution using e.g. the Shapiro-Wilk test can indicate to what degree the sample data is normally distributed and thus if parametric statistics can be used. Log-transformation of a set of data might transform non-normally distributed data into normally distributed data for which

parametric statistics can be used. The term geometric mean is used to describe a mean value originating from log-transformed analysis.

In our analysis of FC values, we have chosen to use parametric statistics after log-transforming our initial data. Initial reports regarding FC levels in IBD and other diseases often used non-parametric statistics or parametric statistics without log-transformation.[238] Recently however, other have suggested that FC is best studied with parametric statistics after logarithm transformation.[239]

Comparative analyses

In this thesis, we have used Student's t-test and 1-way analysis of variance to study differences of normally distributed data among different groups. For data that was not normally distributed, i.e. data obtained from the BLM mouse model, the Mann-Whitney U-test was used.

The statistical analyses in our animal models were challenged by the fact that we had four principally different groups to study; (A) control mice that either were of (1) wild type genotype or were (2) genetically modified, as well as (B) fibrotic mice that either were of (1) wild type genotype or were (2) genetically modified. To verify that the method was working properly, group A1 and B1 were compared. To investigate if the genetically modified animals were different in their physiological state we compared A1 with A2. To finally compare if the fibrotic reaction was different in the genetically modified animals we compared B1/A1 with B2/A2, i.e. we normalised our data from fibrotic mice with the corresponding data from control mice.

Correlation analyses

In our exploration of FC we used Pearson correlation coefficient when comparing log FC with other, normally distributed data. Spearman's correlation coefficient, r_s , was used when FC was correlated with non-continuous variables, e.g. PPI usage.

In our study of the variability of FC, intraclass correlation (ICC) was analysed. This analysis has several similarities with Pearson correlation coefficient, but has some distinctive qualities. Most importantly, the ICC correlates not only the relative distribution within a data set, but do also identify possible systematic differences between two data sets. In our analysis of repeated measurement of FC in SSc, this means that the ICC, but not the Pearson correlation coefficient, would identify any trend of FC at time point 2 to (always) be lower than FC at time point 1.

Generalisability

When presenting our data, standard error of the mean and the p-value, are two ways to indicate the certainty of our descriptive and comparative results. Whether our results are applicable also to other patients or not, depends on the composition and characteristics of the patients studied. Traditionally, as well as in our manuscripts 2, 3 and 4, these data are presented in table 1.

In our epidemiological study, we have not used any descriptive statistics when calculating incidence and prevalence in the Skåne region. It could be argued that such information would be appropriate if one perceives that we have studied a sample population from Sweden or Scandinavia. The pathogenesis of SSc however, is likely to be influenced by yet unknown environmental factors with different geographical distribution. For this reason we have refrained from treating our epidemiological data as a random sample population. Consequently, the calculation of confidence intervals or standard error of the mean regarding our prevalence data has not been applicable.

Ethics

Both the clinical and experimental projects of this thesis were preceded by written permission of the ethical committees at the university.

Human studies

In clinical research, the difference between clinical research and clinical healthcare can sometimes be hard to define. This is often an advantage, especially for the individual patient whose nailfold capillaries could be analysed already 20 years ago as part of on-going research. Nevertheless, according to international and national regulations, this distinction should be made clear in writing. All patients included in paper 2, 3 and 4 gave their written consent to be included in research studies. For patients not being able to give their approval in paper 1, medical records were analysed with the written approval of the owner of these records, i.e. the head of the department.

Animal studies

A central concept in our approved permit for animal studies was the clear definition of study endpoints. Animals fulfilling these endpoints were forced to be sacrificed earlier than planned. This restriction was implemented to avoid

unnecessary animal suffering. This regulation had to be balanced with the fact that we wanted the mice to develop fibrosis and thus to develop a state of at least moderate disease and suffering. Preliminary experiments and contacts with other laboratories gave us important information on BLM dosage in order to make sure that the animals developed skin fibrosis without being seriously ill.

Results

The 2013 classification criteria

Our individual case ascertainment in the Skåne region identified 233 patients fulfilling the 1980 ARA criteria alive in 2010. By applying the 2013 classification criteria the number of patients identified increased by 69 (30 %) to 302. No patient satisfied the 1980 ARA criteria without fulfilling the 2013 criteria.

Patients without skin fibrosis proximal to the MCP-joints satisfied the 2013 criteria by different combinations of available subcriteria. The most common subcriteria satisfied was Raynaud's phenomenon (n=65/69) and sclerodactyly (n=49/69). Nailfold abnormalities were prevalent in at least 39 of the 69 patients. The least common manifestations among these patients were digital tip ulcers and puffy fingers, both prevalent in only 6 of the 69 patients.

Epidemiology of SSc in southern Sweden

Our study of the Skåne Healthcare Register identified 642 subjects eligible for further analysis of medical records. Of these, 302 patients satisfied the 2013 criteria for SSc. The prevalence of SSc was estimated to 305 cases per million adults in the Skåne region 2010. Our data showed a male : female sex ratio of 1 : 6.0. There was a clear dominance of the lSSc disease subset (82 %).

The age distribution is similar to what has been proposed earlier. Notably, the difference between men and women faded out in later ages (Figure 7).

SSc prevalence was also calculated in different counties in the Skåne region (Figure 8).

The annual incidence of SSc in the Skåne region between 2006 and 2010 was 19 per million and year. A majority of these patients had the lSSc subtype. The male : female ratio was 1 : 4.2.

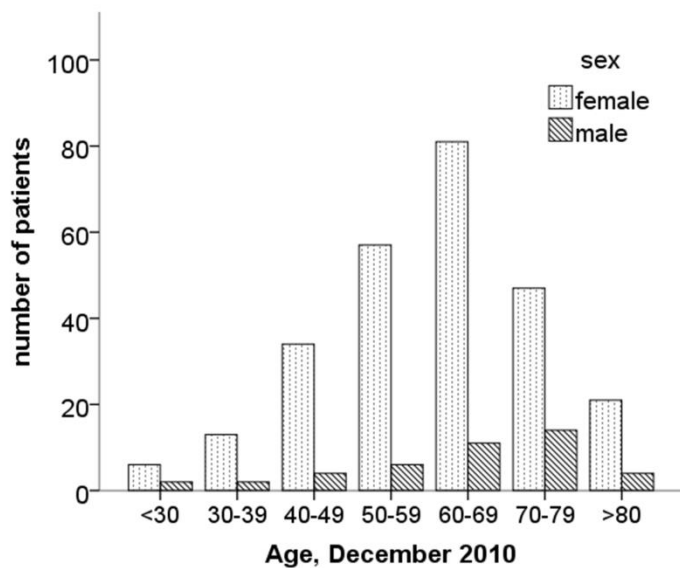


Figure 7. Age and sex distribution of prevalent SSc cases in the Skåne region, 2010
Adapted from paper 1.

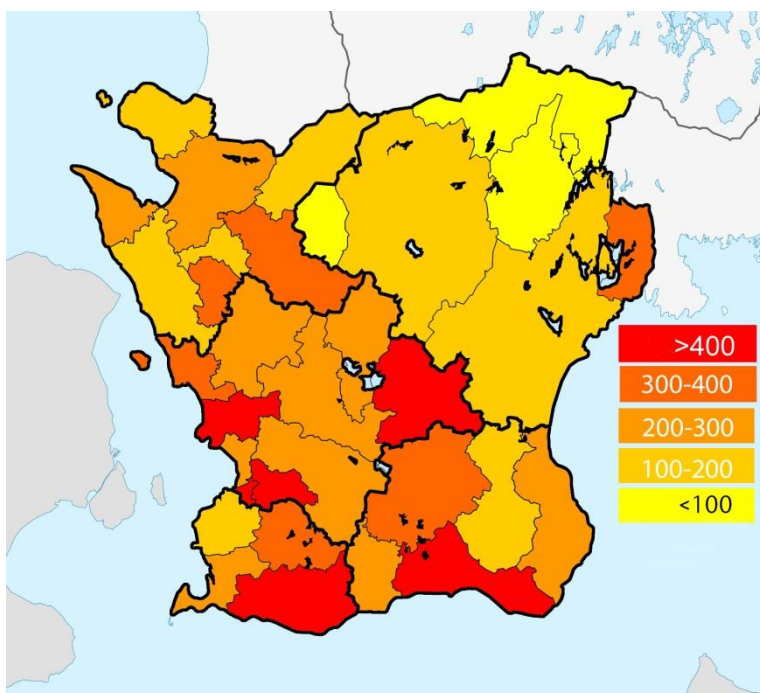


Figure 8. Geographical distribution of prevalent SSc cases in the Skåne region, 2010
Prevalence per million adults.

S-COMP in SSc

Serum levels of COMP predict SSc-related mortality

Among 218 patients followed longitudinally, 84 patients had the dcSSc subtype and 134 had the lcSSc subtype. In total, 70 patients had S-COMP levels above 15 U/l at baseline. Of these, 50 had dcSSc which is in agreement with the fact that skin fibrosis elevates S-COMP. Patients with pathological S-COMP had significantly worse survival rates than other patients (Figure 9). Of the 24 deaths identified in this group, 20 patients had dcSSc of which six patients had fatal ILD. Median S-COMP among these six patients was 20 (range 18-26) at baseline. Among the 148 patients with S-COMP < 15 U/l at baseline, three of the 25 deaths were attributed to ILD.

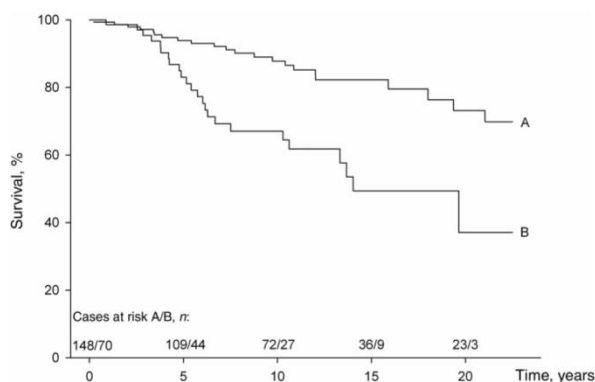


Figure 9. Survival in patients with SSc stratified by S-COMP at baseline

Patients with pathological S-COMP testing (B) have significantly worse survival compared to patients with normal S-COMP (A) ($p < 0.001$). Adapted from paper 2.

Serum levels of COMP and interstitial lung disease

We studied S-COMP in relation to ILD in both a cross-sectional ($n=64$) and a longitudinal ($n=80$) cohort. The cross-sectional cohort consisted of consecutive patients of which 30 had completely normal HRCT. Patients with radiological signs of ILD did not have higher S-COMP than these 30 patients (12.4 vs 12.8 U/l $p=0.707$). Furthermore, the 19 patients with signs of severe ILD on HRCT did not have higher S-COMP values compared to the other patients (11.9 vs 12.8 U/l $p=0.521$).

In the longitudinal cohort we analysed the changes in S-COMP over a period of one year, ($\Delta\text{COMP}_{1\text{year}}$). We also calculated the difference between VC at baseline and the worst VC measurement ever recorded, ($\Delta\text{VC}_{\text{lowest}}$). Finally, we investigated the change in vital capacity (VC) during one year, ($\Delta\text{VC}_{1\text{year}}$). We analysed $\Delta\text{VC}_{1\text{year}}$ as a reflection of on-going ILD at baseline. We chose $\Delta\text{VC}_{\text{lowest}}$ as a marker of final severity of the ILD.

Both $\Delta\text{COMP}_{1\text{year}}$ and COMP at baseline showed a modest but significant correlation with $\Delta\text{VC}_{\text{lowest}}$, ($r_s = -0.29$ and $r_s = 0.23$; $p = 0.011$ and 0.040 respectively). Furthermore, $\Delta\text{COMP}_{1\text{year}}$ also correlated with $\Delta\text{VC}_{1\text{year}}$, ($r_s = -0.32$; $p = 0.005$)

COMP and clinical SSc: conclusions

In this study we also confirmed earlier reports showing a substantial and clinical relevant correlation between S-COMP and mRSS ($r_s = 0.55$, $p < 0.001$, $n = 133$). We extended earlier observations and identified pathological S-COMP as a predictor of mortality in SSc. We also show that increasing S-COMP in early SSc has a limited association with severe ILD. While we conclude that the biomarker potential of S-COMP for SSc lung disease is fair, our results motivated further experimental studies regarding the role of COMP in the development of fibrosis.

Experimental fibrosis in COMP deficient mice

COMP deficient mice are not resistant to skin fibrosis

Skin fibrosis was induced in mice deficient in COMP and wild type mice of the same strain (C57/Bl6) according to the BLM mouse model.

In wild type mice, mean serum levels of COMP after 4 weeks of BLM injections, were not different in BLM and NaCl treated mice (1.23 vs 1.31 $\mu\text{g/ml}$; $p = 0.446$).

Skin fibrosis developed in both wild type and COMP deficient mice (Figure 10). Compared to NaCl injected control mice, median skin thickness increased with 49 % in COMP deficient mice and 56 % in wild type mice ($p = 0.904$; $n = 36$).

Measurement of hydroxyproline content in skin biopsies showed median increase in skin collagen content of 94% in COMP deficient mice and 76% in wild type mice ($p = 0.460$, $n = 41$).

In conclusion, skin fibrosis can develop in mice also in the absence of COMP. I believe further studies are necessary in order to further elucidate the role of COMP in the development of fibrosis.

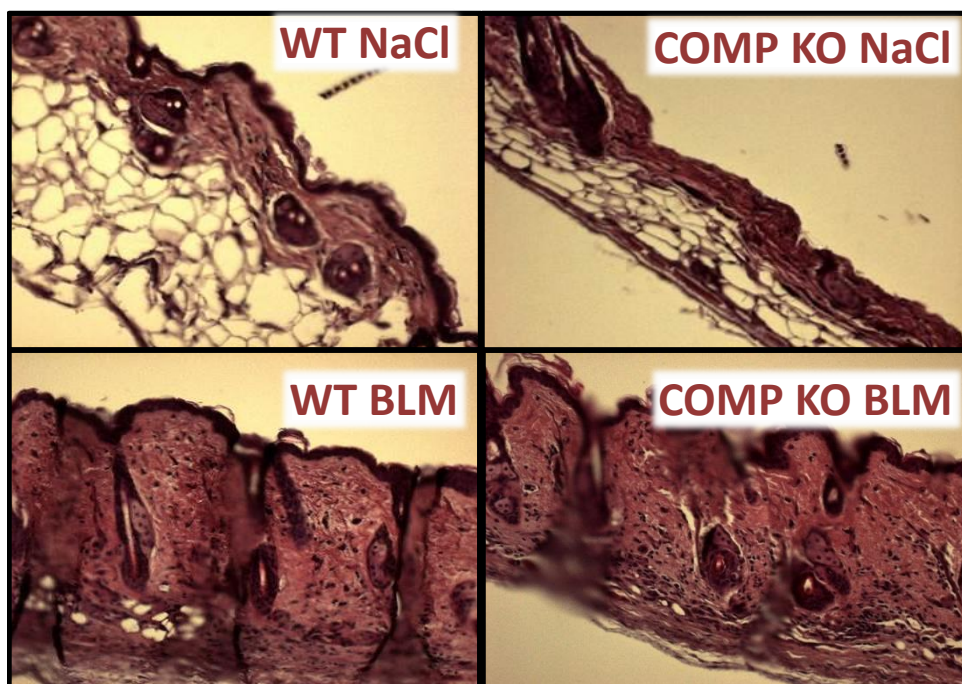


Figure 10. Histological examination of mouse skin subjected to bleomycin (BLM) injections
Skin fibrosis develops both in wild type (WT) and COMP deficient (COMP KO) mice in the bleomycin mouse model. Haematoxylin and eosin staining.

F-calprotectin (FC) in SSc

Patients with SSc have increased levels of FC that correlate with GI disease

In an exploratory, cross-sectional study we investigated FC levels in consecutive SSc patients. We conclude that a majority of SSc patients have pathological levels of FC. Furthermore we identify pathological cineradiography, micronutrient deficiency and a history of referral to another clinic because of GI disease and usage of PPI and other GI medications to be associated with increased levels of FC (Figure 11). We did not find any association between subjective symptoms of GI disease and FC.

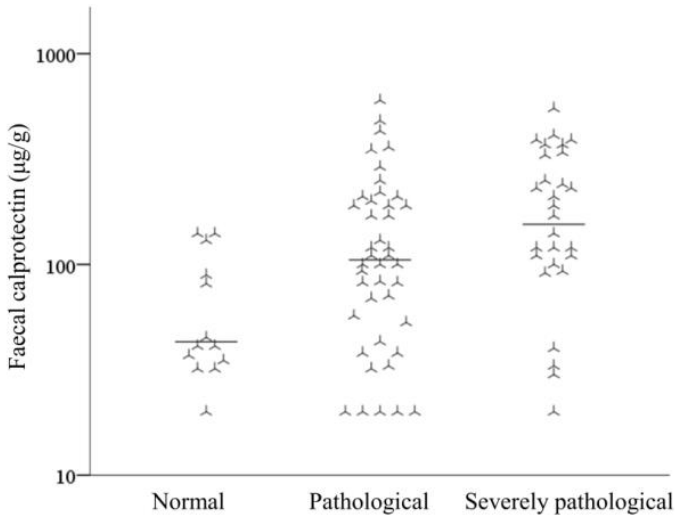


Figure 11. Patients with pathological cineradiography have higher levels of faecal calprotectin than other patients

Adapted from paper 4.

Levels of FC show little variation over time

In a prospective study, FC was analysed in 93 participants at least twice. The average time between the two analyses was 1 year. The consistency of the FC measurement was substantial, ICC=0.69. Furthermore, pathological FC testing was common already in early disease and was not likely to change over time (Table 4).

Variability of FC over time	Typical FC level			All patients, n (%)
	normal, FC < 50, n	elevated, FC = 50-200, n	substantially elevated, FC > 200, n	
Solid	6	7	6	19 (33)
Stable	3	18	12	33 (58)
Variable	0	4	1	5 (9)
All patients	9	29	19	57 (100)

Table 4. Faecal calprotectin (FC) is stable over time in SS_c

Stool samples were delivered for analysis of FC (µg/g) at 3 times and categorised as normal, elevated or substantially elevated as well as solid, stable or variable. Adapted from paper 4.

Levels of FC show little variation upon cessation or initiation of immunosuppression

Immunosuppressive therapy has been shown to modulate lung and skin manifestations of SSc.[58] Given a plausible common inflammatory pathology behind these manifestations and SSc GI disease, we wanted to investigate if immunosuppression would affect FC levels in SSc. We identified 23 cases where FC was measured both with and without immunosuppression. We could not find any association between FC and immunosuppression.

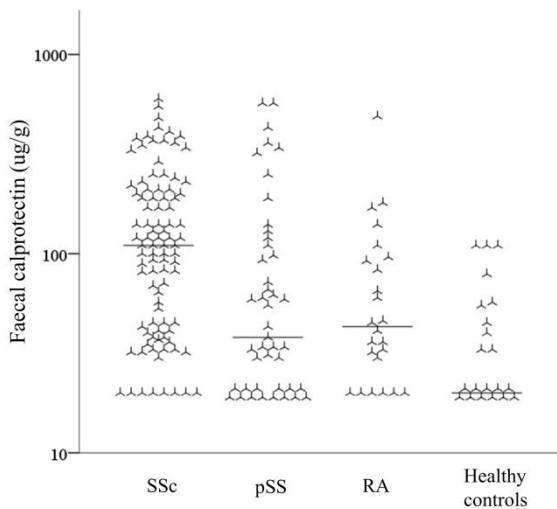


Figure 12. Faecal levels of calprotectin are higher in SSc compared to other rheumatic diseases
Adapted from paper 4. pSS: primary Sjögren's syndrome, RA: rheumatoid arthritis.

Levels of FC are higher in SSc compared to other rheumatic diseases and healthy controls

FC was measured prospectively in patients with RA, primary Sjögren's syndrome (pSS) and healthy controls. FC levels were significantly higher in SSc subjects than any of these groups. Several patients with rheumatic diseases had pathological FC testing. FC values obtained from healthy controls supported that the cut-off value suggested by the laboratory was reasonable (Figure 12).

Experimental fibrosis in S100A9-deficient mice

S100A9 deficient mice are not resistant to skin fibrosis

Skin fibrosis was induced in mice deficient in S100A9 and wild type mice of the same strain (C57/Bl6) according to the BLM mouse model. Animals were sacrificed after 1, 2 and 4 weeks of BLM injections. S100A9 deficient mice have been shown to lack also S100A8.[182] The model was modified in comparison to the COMP-investigation in regard to dosage of BLM, localisation of the injections and shaving routine.

Wild type mice showed extracellular deposition of S100A8 and S100A9 in skin injected with BLM (Figure 13). Skin fibrosis developed in both wild type and S100A9 deficient mice (Figure 14). Compared to NaCl injected control mice, skin thickness increased similarly in S100A9 deficient mice and in wild type mice at 4 weeks, (80 vs 90 % increase, $p=0.760$).

Measurement of hydroxyproline content in skin after 4 weeks of BLM injections indicated a similar increase in skin collagen content in S100A9 deficient mice and wild type mice (41 vs 50 % $p=0.133$).

S100A9 deficient mice show similar inflammatory response as wild type mice in experimental skin fibrosis

To further study the dynamics of inflammation in the absence of S100A8/A9, the number of infiltrating cells were analysed by IHC. Infiltrating T-cells peaked after 2 weeks when it showed a 3.4-fold increase in S100A9 deficient mice and a 2.6-fold increase in wild type mice ($p=0.279$).

Eventually, macrophages infiltrated the dermis. After 4 weeks, the number of macrophages increased 3.1-fold in S100A9 deficient mice and 3.0-fold in wild type mice ($p=0.851$).

Fibroblast transformation into myofibroblasts has been described in both SSc and the BLM mouse model, and is thought to contribute to fibrosis development. Myofibroblasts were identified with positive IHC staining of smooth muscle actin and typical spindle like shape. Myofibroblast infiltration peaked after 2 weeks. S100A9 deficient mice showed a 4.3-fold increase while wild type mice showed a 3.4-fold increase ($p=0.445$).

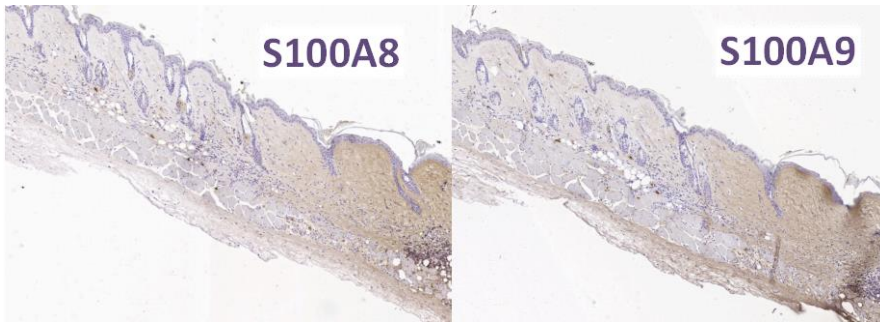


Figure 13. Localised deposition of S100A8 and S100A9 in skin injected with BLM
Sequential sections from fibrotic and inflamed skin in wild type mice treated with BLM for 2 weeks. Immunohistochemical analysis of S100A8 and S100A9. Adapted from paper 5.

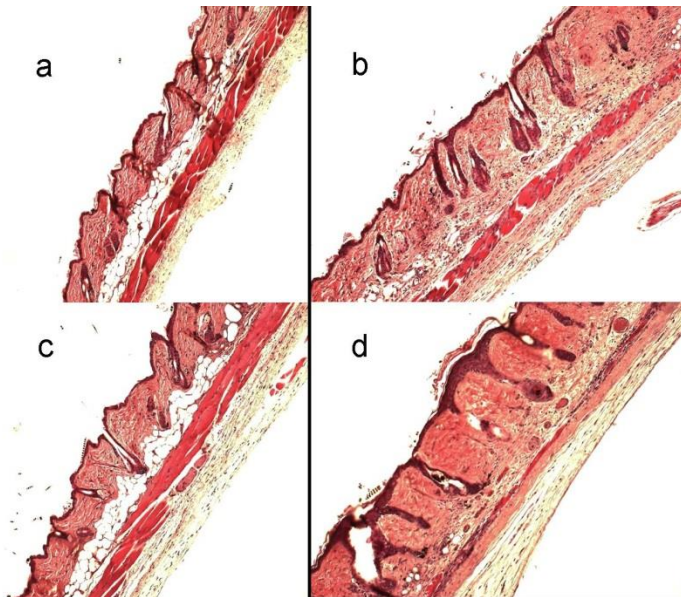


Figure 14. Skin fibrosis develops both in wild type mice and mice deficient in S100A9
(a) wild type control, (b) wild type bleomycin (BLM), (c) S100A9^{-/-} control, (d) S100A9^{-/-} BLM. Both wild type and S100A9^{-/-} mice treated with BLM showed dermal thickening, epidermal hypertrophy and subcutaneous fat atrophy. Haematoxylin and eosin. Adapted from paper 5.

Discussion

Classification and diagnosis of SSc

After 33 years, the 1980 ARA preliminary criteria have now been replaced by a new set of criteria. We used both the 1980 ARA and the 2013 classification criteria in an unselected population. We demonstrated that the new criteria identify subjects who otherwise would not fulfil the 1980 ARA criteria and showed that a typical patient identified with these criteria was a woman with ISSc suffering from Raynaud's phenomenon and sclerodactyly who had antibodies against centromeres. A similar disease subtype was described in 1959 by Hans Langgård. Interestingly, the first patient presented in his original article does not satisfy the 1980 ARA criteria. Yet it is uncontroversial to agree with Langgård's conclusion that these patients represent a subtype of scleroderma, a conclusion that the local doctor in the small town of Thisted made already in 1947. The author further explains:

Noget endeligt navn vil sygdommen næppe få, før den er blevet placeret aetiologisk, patogenetisk og i relation till de øvrige kollagen-sygdomme.

This disease is unlikely to receive a final name, until it has been correctly placed in reference to aetiology, pathogenesis and in relation to other rheumatic diseases. [my translation]

According to Langgård's case report, the patient might satisfy the 2013 criteria, suffering from sclerodactyly, Raynaud's phenomenon and a history suggestive of digital ulcer (Figure 15). As of this year, Langgård's patients would finally "receive a name" and be classified as SSc.

The 2013 classification criteria acknowledge not only the value of proper assessment of skin thickness, but also the clinical value of nailfold capillaroscopy, HRCT, invasive diagnosis of PAH and screening of RNA polymerase III-antibodies. All these methods have been a part of routine clinical care at the SSc centre in Lund for several years, especially in the diagnostic evaluation of patients with suspected SSc. This fact increases the validity of our application of the 2013 classification criteria.

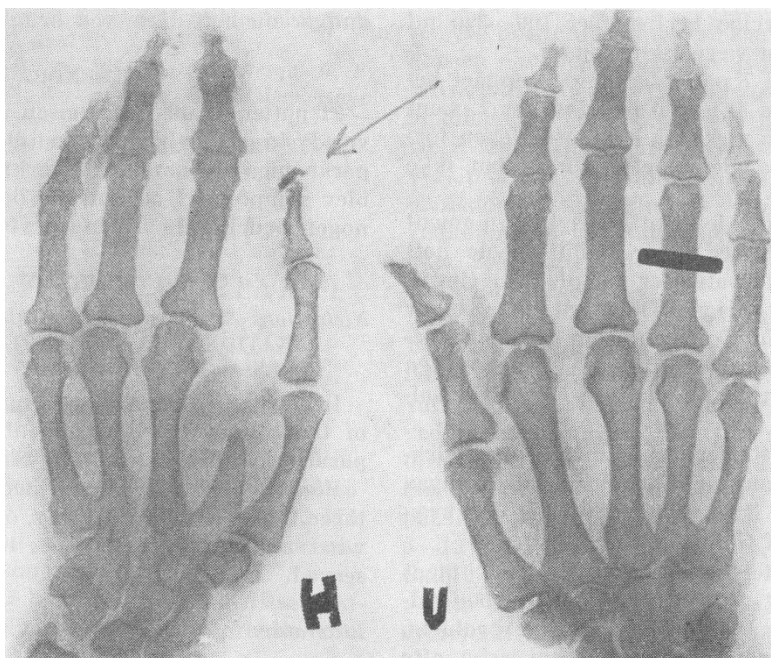


Figure 15. X-ray of the hands of a patient suffering from SSc in 1947

Notice the amputated right index finger (H). This patient did not satisfy any SSc criteria before 2013.[7, 11]

The development of the 2013 classification criteria was a joint venture between the ACR and the EULAR. This process involved 41 of the major SSc centres in America and Europe. It is a strength of our study that the SSc centre in Lund was not involved in this development process. Our routine clinical care has remained unbiased and independent from the development process of the 2013 classification criteria until 2012.

Prevalence of SSc

Both the U.S. and the European Union have acknowledged that rare diseases might be neglected by the pharmaceutical world. In its own words, the U.S. States Drug Act expresses that rare diseases

”for which there is no reasonable expectation that the cost of developing and making available in the United States a drug for such disease or condition will [be] recovered from sales in the United States of such drug”[240]

can be referred to as **orphan diseases**. In order to be classified as an orphan disease, prevalence estimates must be below 670 or 500 per million according to

the U.S. and the European Union respectively.[241] Our data supports previous reports indicating that the 1980 ARA criteria have underestimated the true prevalence of SSc.[12] Our investigation does however also support the notion that the current classification of SSc as an orphan disease is correct.

The geographical distribution of SSc has been subject to multiple discussions.[21, 242] Our results do not support the concept of a previously suggested north-south gradient of SSc in Europe.[18] It may be tempting to hypothesise on possible environmental factors contributing to SSc development based on figure 8 presented in this thesis. I believe that interpretations from this figure should be made with caution since several of the counties in the Region have a population less than 10 000. Furthermore, the age distribution varies between different counties. Our study showed that a majority of the SSc patients alive in 2012 were above 60 years of age. While 46 % of the population is above 50 years of age in Ystad county, only 31 % of the population is above 50 years of age in Lund county.[243]

Until 1999, healthcare in the north-eastern part of the Skåne region was administratively independent from the south-west, in what was called Kristianstad läns landsting. One could speculate if this history, together with the geographical distance, can have resulted in less frequent SSc inquiries from this region reaching the SSc centre in Lund, and eventually, e.g. capillaroscopic examination. Such a hypothesis might be supported by the distribution of SSc prevalence presented in figure 8.

Novel biomarkers in SSc

Already 50 years ago, the medical statistician Donald Mainland stated:

Will the variables observed be the variables that we really wish to know about? If we are substituting something that is easy to observe for something that is difficult to observe, we have no right to do so unless we know the connection between the two things.[244]

This statement might seem self-evident, but encompasses several of the aspects in biomarker development and validation that has now been formalised and carefully defined through the continuous development of the OMERACT concept of truth, discrimination and feasibility.[123]

S-COMP

Concerning S-COMP, independent studies have shown a statistical connection between the biomarker and mRSS. These findings are supported by molecular, biochemical and immunohistochemical data showing a clear anatomical connection between mRSS and S-COMP in the affected skin.[139, 148] Furthermore, our current knowledge of COMP and collagen fibrillogenesis has revealed a mechanistic association between COMP and sclerodermatous skin transformation.[146] The relation between S-COMP and skin fibrosis is more than a statistical association, we have sound knowledge regarding this “connection”.

From another perspective, Mainland’s comments might challenge the biomarker potential of S-COMP and SSc. Is the skin fibrosis the primary concern for the SSc patient? Is treatment of skin fibrosis the main unmet need in SSc? Is the historical focus on skin manifestations of SSc – described already more 150 years ago – reflecting the patient’s SSc-related sufferings or the clinical characteristics that are most evident for the examining physician?[245]

For some patients, skin fibrosis is definitively the main suffering. But for patients suffering from severe ILD or renal involvement, the situation may be very different. In our communication in *Rheumatology*, these questions become important as we analyse associations between S-COMP and mortality. We identified an association between S-COMP and SSc prognosis, but the mechanisms involved – the nature of the connection - remain largely unexplored. One could ask if S-COMP is a biomarker for another biomarker – the mRSS? Larger prospective studies are needed to further investigate these associations if we are to follow Mainland’s advice. Alternatively, it can be argued that even if COMP is not directly involved in the fatal pathologic processes of SSc, the predictive value of COMP remains (Heinegård D., personal communication).

Our reported data on S-COMP and pulmonary function tests and HRCT gives us new insight in the origin of S-COMP in SSc. Our results suggest that active pulmonary fibrosis does not affect S-COMP to any large degree. From a biomarker perspective, this might be an advantage, reducing the complexity of interpreting S-COMP in SSc. Furthermore, it indicates a qualitative difference between COMP and procollagen III N-terminal propeptide – the later one showing correlations both to skin and pulmonary fibrosis.[246] Our results might be regarded as unexpected considering earlier reports of COMP expression in lung tissue.[148] There are several possible explanations for these results. One possibility would be that the mere size of fibrotic skin exceeds that of pulmonary fibrosis, resulting in S-COMP mainly reflecting fibrotic processes originating in the skin. In addition however, it should be acknowledged that we do not know what processes the pentameric COMP-molecule is subject to before it reaches the serum where it is measured. The ELISA used in this study uses two monoclonal

antibodies both recognising different parts of the stalk region in the COMP molecule which not necessarily need to be pentameric.

Several reports indicate that the inter-individual variation in S-COMP is substantial while the intra-individual variation in healthy subjects is limited.[165, 247] Analogous with pulmonary function tests, a single analysis of S-COMP might thus be hard to interpret. In order to appreciate the significance of one single measurement that is only slightly outside the reference values, it would be ideal to know what levels the patient presented before the onset of SSc. This would be especially true for SSc-patients with co-existent OA.

One method of adapting to this situation is to analyse Δ S-COMP. In RA, this measurement has been shown to be a better indicator of active disease processes than individual S-COMP measurements.[247] Patients with newly diagnosed dSSc are often subject to multiple contacts with healthcare during a short period of time and evaluation of Δ S-COMP would thus still be a feasible analysis. Our report indicates that Δ S-COMP might be more informative than single S-COMP measurements. For future explorations of the biomarker potential of S-COMP in SSc, it is possible that studies of Δ S-COMP might be most fruitful.

Faecal calprotectin

In the field of SSc GI disease, Mainland's aforementioned quote regarding the necessity to "know the connection between the two things" is even more challenging. SSc GI disease is heterogenous. Several of these GI manifestations might be inconspicuous for the patient yet of substantial clinical importance, e.g. slowly progressing anaemia, malnutrition and asymptomatic oesophageal reflux disease. So far, we have only discerned a statistical association between SSc GI disease and the biomarker FC. This association is also yet to be proven by other, independent investigators. Still, this connection seems to be stable over time and could thus warrant further studies regarding a plausible mechanistic relationship between FC and SSc GI disease(s). I agree with Mainland that I would not choose FC as an alternative for a thorough clinical evaluation coupled with necessary invasive investigations. Rather, I would like to propose FC as a promising complement to the most delicate issue of objective GI assessment.

I doubt FC is yet to serve such a role in the individual clinical evaluation of SSc. I am, however, inclined to believe that the situation is different in clinical trials of SSc. The fact that we do not know any drug or treatment that counteract the basic pathologies in SSc GI disease does not exclude the possibility that such a treatment might already exist. It can be speculated that novel treatment regimes for other SSc manifestations, such as endothelin receptor antagonists and autologous stem cell therapy, might also attenuate or reverse GI pathology in SSc, although we are not (yet!) aware of it. In order to prove or disprove such a hypothesis,

invasive measures are needed. As an initial alternative approach, I suggest that the addition of FC as secondary or tertiary end-point in future clinical trials could indicate if a treatment warrants further exploration regarding its GI effects.

The University of California, Los Angeles Scleroderma Clinical Trial Consortium GI Tract 2.0 is a questionnaire specifically developed for the assessment of SSc GI disease. It has been shown to be reliable, valid and feasible and has been proposed to be a core set response measure in the assessment of SSc GI disease.[222, 248] It has shown moderate associations with objective investigations of GI disease.[223] As mentioned earlier, also SSc patients without GI symptoms might suffer from SSc GI disease.[95] Similar to IBD, there is a discordance between patient reported symptoms and results from objective investigations.[110] I suggest that in addition to invasive measures and patient reported outcomes, the area of soluble biomarkers, such as FC, warrants further exploration in SSc.

Such explorations should hopefully be able to answer questions regarding the origin of FC in SSc. Since SSc can affect almost any part of the GI tract the possible contribution from the oesophagus and the ventricle as well as the small and large intestine remains to be elucidated. Furthermore, in SSc we do not know from what cells FC originates. Although conclusions made from studies in IBD indicate that neutrophils or at least leukocytes are the main contributors, we cannot rule out a possible contribution from the intestinal epithelium.[189]

Animal studies

Experimental models of diseases offer a multitude of investigational possibilities compared to human studies. Importantly, the organism can be studied during the initiation steps of disease, affected tissues can systematically be dissected and analysed, environmental factors can be minimised (or at least standardised) and the natural course of the disease be studied without pharmaceutical intervention.

Animal models might both give information on the pathological mechanisms of disease as well as indicate the possible effect and safety of novel treatments. In the first case, the usage of genetically modified mice has been indispensable. In the field of rheumatology, the introduction of biological therapy in the late 1990's was preceded by experimental studies in both human samples and in genetically modified mice.[249, 250]

In view of the many advantages offered by animal studies, the limitations of such investigations in general and of BLM models and other models of autoimmune diseases in particular, should also be mentioned.

In reference to inflammatory conditions, it has been suggested that murine models are quite different from human pathologies. In 2004, in their article *Of Mice and Not Men: Differences between Mouse and Human Immunology* the authors stated:

Mice not only live in different ecological niches, they are also much smaller and have significantly shorter lifespans. These are not trivial differences.[251]

Recently, a systematic comparison between human and murine inflammation in reference to total gene expression in leukocytes was presented. The study focused on three different clinical conditions (burns, trauma and sepsis) and their corresponding mouse models. This multicenter project identified changes in 4918 human genes which all had murine orthologs. Surprisingly, the three human conditions showed little differences between each other in reference to molecular pathways used. In contrast, these conditions showed very weak molecular similarities with their corresponding animal models. The authors conclude that, at least for inflammatory conditions, mouse models are in many cases not reflective of human conditions on a molecular level.[252]

Differences in human and murine immunology may in part explain previous results from the BLM mouse model of lung fibrosis. In 2008, 246 compounds that had shown promising results in the BLM mouse model of lung fibrosis were reviewed. None of these compounds had at that time qualified for clinical use in humans.[253] One major concern for the author was to differentiate between BLM induced inflammation and fibrosis, since the latter is not always a consequence of the first. This is a complex issue that we have studied further.[254] One of the more promising candidates in the BLM review, the tyrosine kinase inhibitor imatinib, has also been studied in SSc. Promising results from several animal models of SSc could not be repeated in a randomised double blind phase II trial.[255]

SSc is a complex disease involving fibrosis, vascular dysfunction and autoimmunity. Several animal models have been suggested to reflect these pathologies. The BLM model of skin fibrosis definitively exhibits both fibrosis and inflammation. It has also been suggested that the inflammation is associated with autoimmunity.[227] The model does however not develop any vascular changes typical of SSc. Furthermore, the model does not include any gut pathology as we are aware of today. This is a limitation in our studies on both COMP- and calprotectin deficient mice.

Several other animal models for SSc have been presented. From the perspective of calprotectin, it is interesting to note that fibrosis of the gut has actually been demonstrated in some of these models. In 2012, Thouta et. al., elegantly demonstrated that the TBRII Δ k model does not only develop skin, lung and vascular pathologies resembling SSc. Histological and molecular analysis together with physiological examinations revealed that these mice develop intestinal fibrosis and have pathological contractility of the gut, resembling SSc.[256] In

2009, mice with overexpression of PDGFR α were shown to develop fibrosis in multiple tissues including the intestine.[32] Some reports of the well established murine chronic graft vs host animal model of SSc have indicated that also these mice may develop intestinal fibrosis.[257] Finally, an avian model of SSc, the UCD-200 chicken should be mentioned. Not only do these chickens develop endothelial apoptosis, cellular infiltrates and fibrosis in the oesophagus – they also show signs of systemic vascular damage, inflammation, autoimmunity and fibrosis.[258, 259] Usage of this animal model has been rather limited, possibly because of practical demands in housing and breeding these birds, as well as the limited tools to study the fibrosis, immunity and the vascular system in an avian system.[260]

It is possible that further animal studies using these models might give further insights in the origin of FC in human SSc. It can also be argued that the structural and functional differences between human and murine inflammation, fibrosis and function of S100 proteins might decrease the clinical significance of such investigations. Consequently, further human studies may be more informative than any further animal model.

COMP

Structural similarities between COMP and other TSPs may indicate functional redundancies of these proteins. While COMP is the only TSP found in cartilage, other TSPs – not at least TSP-1 – are present in fibrotic tissues. Studies in rodents have indicated an essential contribution of TSP-1 to the development of lung fibrosis. Treatment with TSP-antagonists ameliorated BLM induced lung fibrosis in rats. In contrast, mice deficient in TSP-1 were not resistant to BLM induced lung fibrosis.[261] This finding is somewhat surprising considering the fact that TSP-1 deficient mice have a phenotype similar to TGF- β deficient mice.[262]

Our current knowledge on COMP indicates a crucial role in the fibrillogenesis and in the molecular arrangement and structural integrity of the ECM. It could be argued that COMP deficiency might alter the quality rather than the quantity of collagen in the fibrotic skin. It could also be argued that COMP deficiency would affect the rate by which immature collagen is arranged into collagen mature fibers. We have neither studied the qualitative characteristics nor the temporal aspects of skin fibrosis in COMP deficient mice. Thus, several aspects on COMP function and fibrosis development remain to be explored. COMP deficient mice have been used in other animal models. In a study of collagen induced arthritis, these mice developed more severe arthritis. In another model of arthritis, the collagen antibody-induced arthritis, COMP-deficient mice did not react differently compared to wild type mice.[263] If further experimental mouse studies using the

BLM or alternative models for SSc is the most appropriate and feasible way to understand COMP in relation to SSc is not self-evident at the moment.

Calprotectin

The many roles of the S100-proteins in general and the calprotectin dimer in particular are yet only partly revealed. While initially thought to reflect mainly inflammation and to originate only from inflammatory cells, novel investigations have challenged this concept. From a SSc perspective, it is thought provoking that a murine study showed that S100A8 can be induced in fibroblasts following stimulation with fibroblast growth factor-2 *in vitro*. This response could be enhanced by addition of heparin but suppressed by TGF- β . In a rat model of wound healing, increased expression of S100A8 was seen in dermal fibroblasts.[190] S100A8 has not been shown to be expressed in human dermal fibroblasts, but synovial fibroblasts from RA and OA patients have been shown to express both S100A8 and S100A9.[178, 264]

The S100 proteins show moderate homology between humans and mice. While glucocorticoids suppress S100A8 and S100A9 in mouse skin, glucocorticoids have been shown to upregulate these proteins in human monocytes. S100A12 is absent in mice. It has been suggested that some of the effects mediated by S100A8 in mice are mediated by S100A12 in humans.[178]

Calprotectin acts as a DAMP on the TLR4. In a murine model of BLM induced lung fibrosis, deficiency of TLR4 was associated with worsening of both inflammation and fibrosis.[265] On the contrary, studies on human samples have indicated that TLR4 activation can stimulate ECM deposition and thus contribute to the development of SSc.[45] In summary, it has been concluded:

The differences in expression patterns of murine and human S100A9 in activated monocytes/macrophages indicate important functional differences and question the roles of the S100A8/A9 complex in chronic inflammation in mice[178]

Increased expression of calprotectin has been shown in BLM induced lung fibrosis.[266] Our study show that both S100A8 and S100A9 are expressed in BLM induced skin fibrosis. Still, however, our study indicates that both the BLM induced inflammation and the BLM induced fibrosis are quite similar in wild type and S100A9 deficient mice. This indicates that processes independent of S100A8 and/or S100A9 may induce and propagate fibrosis in mice. This might be of value considering current projects focusing on pharmaceutical intervention in SSc by targeting the calprotectin complex.[267]

Conclusions

In reference to the aims presented I would like to conclude:

- The 2013 classification criteria for SSc identifies SSc patients not previously identified with the 1980 ARA criteria
- The prevalence of SSc in the Skåne region is higher than previously reported from northern Europe
- The biomarker S-COMP has predictive potential for survival in SSc
- In mice, skin fibrosis can develop also in the absence of COMP
- FC is a potential biomarker of SSc GI disease
- FC is a reliable biomarker in SSc
- In mice, skin fibrosis and skin inflammation develops also in the absence of S100A9 in response to BLM injections

Future perspectives

In reference to the presented data I would briefly like to mention some possible future perspectives based on my studies.

- As a direct consequence of the study on SSc epidemiology I suggest that procedures previously only used for research purposes, e.g. capillaroscopy and some immunological tests, now should become clinical routine.
- Research centres outside Lund should validate the S-COMP results from Lund. The biomarker potential of S-COMP can be further explored by analysing Δ COMP prospectively. Optimally, I would also like to examine S-COMP among patient with late dcSSc - to investigate if COMP is normalised over time in patients with skin fibrosis in regress.
- There is an unmet need for objective yet non-invasive biomarkers for GI manifestations of SSc. Further studies on the possible contribution of calprotectin to SSc-specific pathology are warranted. Molecular studies of GI biopsies from SSc subjects are one possibility. Investigation of FC in comparison to invasive measures such as oral-caecal transit time, another.
- Given the contribution of vascular dysfunction in GI pathology, I suggest that future trials of vascular therapy in SSc should include FC as a secondary or tertiary variable.
- In the BLM mouse model, deposition of S100A8 and S100A9 should be analysed in reference to TLR4 expression.

Populärvetenskaplig sammanfattning

Systemisk skleros (SSc) är en relativt ovanlig reumatologisk sjukdom som kan engagera många av kroppens organ. Sjukdomen kallas ibland också för sklerodermi. Typiskt för sjukdomen är att huden omvandlas till fibros och blir hård och stram över olika delar av kroppen. Typiskt för sjukdomen är också att drabbade patienter har försämrad funktion i sina blodkärl, framförallt i händerna. Vid kyla och annan stress utvecklar händerna därför ofta smärtsamma färgskiftningar i vitt, blått och rött. Vissa patienter kan också utveckla svårläkta sår.

SSc brukar drabba även andra delar av kroppen än huden och händerna. Av denna anledning kallas sjukdomen just *systemisk* skleros. Bland annat har nästan alla patienter med systemisk skleros något besvär ifrån mag- tarmkanalen - mest typiskt är svårbehandlade sura uppstötningar. Om SSc drabbar hjärta och lungor kan den ofta orsaka för tidig död.

För att klassificera patienter med SSc har forskare traditionellt använt sig av kriterier som utarbetades för mer än 30 år sedan. Dessa kriterier fokuserar främst på huden och händerna – på det kanske mest iögonfallande vid SSc - patientens fibrotiska utsida. Nyligen har nya kriterier för sjukdomen föreslagits. Dessa tar också hänsyn till andra typiska kännetecken för SSc som inte är direkt kopplade till hudfibros, t.ex. antikroppar i blodet. Med hjälp av dessa kriterier kan både patienter med tidig sjukdom och patienter med allvarliga manifestationer i hjärta och lungor – men med sparsam hudfibros – korrekt klassificeras som SSc-patienter.

I vårt första arbete har vi studerat förekomsten av SSc i Skåne enligt både gamla och nya kriterier med hjälp av ett unikt sjukvårdsregister och noggrann journalgenomgång. Vid utgången av 2012 var antalet patienter med SSc i Skåne 302 enligt de nya kriterierna (och 233 enligt de gamla kriterierna). Båda dessa siffror är högre än vad som tidigare rapporterats från Danmark, Norge, Finland, Island och England, men påminner om rapporter från södra Europa. Våra resultat kan också uttryckas som att systemisk skleros är en så pass vanlig sjukdom att, i genomsnitt, någon är drabbad i varje ort med åtminstone 4 000 invånare. Vår undersökning indikerar också att de nya kriterierna är bättre än de tidigare för att korrekt klassificera patienter med sparsam hudfibros eller tidig sjukdom.

Den fibrotiska vävnaden vid SSc innehåller en stor andel kollagen. Cartilage oligomeric matrix protein (COMP) är en molekyl som hjälper kollagentrådar att "flätas samman" i starka fiber. Stora mängder COMP kan ibland påvisas i blodet hos patienter med SSc, särskilt hos de med mycket fibros i huden. Vi har undersökt detta protein hos patienter med SSc och samtidig lungfibros. Till skillnad från hudfibros kunde vi inte hitta ett tydligt samband mellan lungfibros och nivåer av COMP i blodet. Vi kunde däremot visa att SSc-patienter med förhöjda nivåer av COMP i blodet har en ökad risk att dö i förtid.

Jämfört med huden, men också lungorna, är mag- tarmkanalen ett svårundersökt organ. Därför är det svårt att veta om t.ex. modern medicinering kan påverka SSc-sjukdomen i denna del av kroppen. Ett sätt att förstå mag- tarmkanalen vid SSc är att genomföra invasiva undersökningar, t.ex. gastroskopi. En annan rekommenderad metod är att be patienten fylla i formulär med riktade frågor om symptom från denna region. Båda dessa metoder har sina fördelar, men också nackdelar. T.ex. kan en gastroskopi ofta vara smärtsam, medan en patients beskrivning av sina symptom i ett formulär kan präglas av andra faktorer än sjukdomen, såsom rädsla och förhoppningar inför framtiden.

Vi har undersökt möjligheterna att analysera sjukdomsaktivitet i mag- tarmkanalen genom att mäta ett protein, kalprotektin, i avföringen. Vi visar hur nivåer av detta protein korrelerar med andra mått på SSc-typisk sjukdom i denna region, t.ex. malnutrition och resultat från sväljningsröntgen. Vi visar också hur fekala nivåer av kalprotektin visar begränsad variation vid upprepad provtagning och att patienter med andra reumatiska sjukdomar har lägre nivåer av detta protein. Vi föreslår att fekalt kalprotektin kan utgöra ett komplement till invasiva undersökningar vid uppföljning av patienter med SSc.

För att förstå vilken roll kalprotektin kan spela i utvecklingen av fibros har vi undersökt fibrosbildning och inflammation i en djurmodell. Vi visar att kalprotektin ansamlas i inflammerad och fibrotisk musvävnad. Vi visar också att möss som saknar kalprotektin inte är skyddade från inflammation och fibros i vår djurmodell.

Sammanfattningsvis har vi visat på en högre förekomst än tidigare beskrivits för en allvarlig reumatisk sjukdom i södra Sverige. Vi har även föreslagit hur ett blodprov och ett fekalt prov har potential att underlätta den kliniska bedömningen av denna ofta svårvärderade sjukdom.

Acknowledgements

The work presented in this thesis is the result of many people working together. I would especially like to mention

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